Minireview

Endocytosis and signaling cascades: a close encounter

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Abstract Internalization of receptors and other cell surface components is well known to occur via clathrin-mediated endocytosis, although other less well characterized pathways are also involved. Internalized receptors are then delivered to early endosomes, where they are sorted to be recycled back to the plasma membrane for reutilization or transported to late endosomes/lysosomes for degradation. Endocytosis has long been considered as a constitutive, housekeeping function of animal cells that occurs independently of the cellular environment in contrast to regulated secretion. Here, we will discuss recent studies that are uncovering the existence of cross-talk between signaling molecules and components of the transport machinery, indicating that endocytosis can be modulated by signaling pathways.

Key words: Signal transduction; Traffic; Rab protein; Phosphoinositide; Growth factor; Clathrin; Actin; FYVE protein

1. Introduction

By far the best characterized route of internalization into animal cells is the clathrin-dependent pathway, which mediates delivery of endocytosed cell surface components and solutes to early endosomes. Then, molecules that need to be reutilized, including receptors with housekeeping functions, are recycled back to the plasma membrane. In contrast, molecules that need to be degraded, like downregulated growth factor and hormone receptors, are transported to late endosomes and lysosomes. It has long been appreciated that ligand binding controls the internalization and subsequent trafficking of downregulated receptors [1], and signaling may itself be regulated by receptor trafficking [2]. During downregulation, the notion that prevails is that the number of transport vesicles is not changed, but that ligand binding triggers incorporation of receptors into pits that form constitutively at the plasma membrane. Recent studies, however, indicate that Nef-mediated downregulation of cell surface CD4 molecules occurs via a selective population of clathrin-coated vesicles [3]. In fact, it is becoming apparent that internalization pathways can be tuned by signaling cascades. In addition, the function of a number of molecules involved in trafficking appears to be regulated by post-translational modifications (mainly phosphorylation). A complex network of protein–protein interactions is also being revealed at the interface between signal transduction and endocytic traffic, supporting the notion that trafficking and signaling are integrated at the mechanistic level. Finally, clathrin-independent routes of entry also exist, and these can be modulated, at least in part, by signaling cascades. In this review, we will focus on the cross-talk between signaling cascades and endocytic traffic along clathrin-dependent and -independent pathways (Fig. 1).

2. Clathrin-independent endocytic pathways

Evidence is accumulating that internalization can occur via pathways that do not depend on clathrin [4]. Inhibition of the clathrin pathway rapidly stimulates other internalization routes, perhaps as a compensatory mechanism [5–7]. However, some components may be shared by clathrin-dependent and -independent internalization pathways, including dynamin and synaptophysin [8,9]. Another dynamin- and clathrin-independent pathway may also exist [10]. One possible route of entry involves cell surface microdomains containing glycosphingolipids and cholesterol (rafts), which are believed to play an important role in signaling and have a capacity for internalization [11–13]. In fact, recent studies reveal that the interleukin 2 receptor, in contrast to most known receptors that are endocytosed via the clathrin pathway, is internalized via a clathrin-independent route that may involve these microdomains [14]. Rafts can form pear-shaped invaginations termed caveolae, when associated with caveolin [15], and these seem to mediate endocytosis in endothelial cells, and perhaps in other cell types [8,16].

Macropinocytosis is a non-clathrin route of entry related to phagocytosis and associated with areas where membrane spreading and ruffling take place. This route is modulated and used by some pathogenic bacteria [17–19]. Macropinocytosis is stimulated by the growth factor EGF [20] and by Ras [21], the downstream regulator of the mitogenic response, but presumably not in itself by endocytosis of occupied EGF re-

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Abbreviations: PI, phosphatidylinositol; PI(3)P, phosphatidylinositol-3-phosphate; PI(3,5)P_2, phosphatidylinositol-3,5-bisphosphate; PLD, phospholipase D; GAP, GTPase-activating protein; MVB, multivesicular body; ECV, endosomal carrier vesicle

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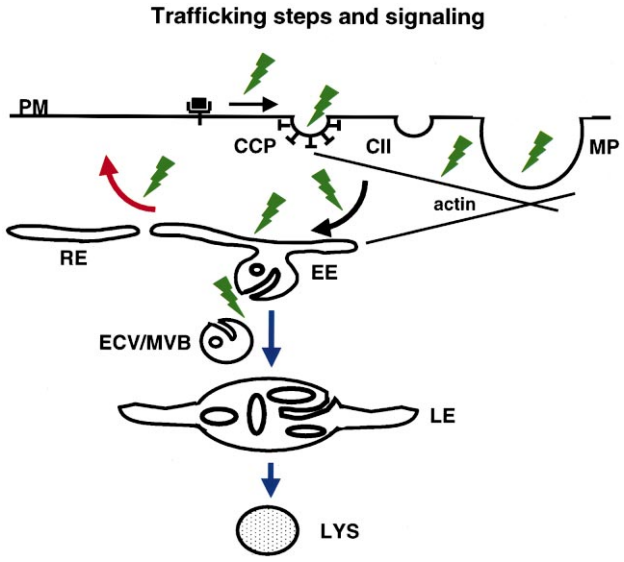

Fig. 1. The pathways of endocytic traffic are outlined on the figure. Internalization occurs via clathrin-coated pits and vesicles (CCP) and via other pathways, including clathrin-independent invaginations (CII) and macropinosomes (MP). Molecules internalized via the clathrin pathway, and perhaps also via other pathways, then appear in early endosomes (EE). From there, some components are recycled back to the plasma membrane (PM), directly from EE or indirectly via recycling endosomes (RE). Molecules destined to be degraded are collected within transport intermediates (MVBs or ECVs) and then are transported to late endosomes (LE) and lysosomes (LYS) for degradation. Lightning arrows point at trafficking steps believed to be regulated by signaling cascades.

ceptors [22]. Vesicles formed when membrane ruffles, presumably macropinosomes, move at the tips of actin tails [23], and macropinocytosis, much like phagocytosis, involves remodeling of the actin cytoskeleton. This process depends on coronin in *Dictyostelium* and perhaps in mammalian cells [24], and on signal transduction pathways involving Rho GTPases (Rho, Rac and Cdc42) [25]. Rho GTPases also appear to be involved in macropinocytosis downregulation during dendritic cell maturation [26]. Both activated RhoA and Rac stimulate pinocytosis, but inhibit clathrin-dependent endocytosis [27,28]. Recent studies also indicate that the p21-activated kinase PAK1, a target for activated Rac and Cdc42 regulating the actin cytoskeleton, is also involved selectively in macropinocytosis [29]. Constitutive ruffling and macropinocytosis in oncogene-transformed fibroblasts appear to depend on activation of both phosphatidylinositol-3-kinase (PI3K) and phosphatidylinositol (PI)-phospholipase C, leading to a reorganization of the actin cytoskeleton [30], in agreement with previous studies indicating that PI3K is involved in macropinocytosis [31].

3. Actin and signaling in endocytosis

It is becoming clear that the actin cytoskeleton plays a general role in endocytosis, in addition to macropinocytosis [28,32], but its precise role remains unclear. Signaling-dependent reorganization of the actin cytoskeleton may contribute to the spatial or temporal regulation of trafficking routes [33]. Although the precise role of the small GTPase ARF6 is still unclear, evidence suggests that it plays a role in some recycling step as well as in cell spreading and adhesion [34,35], maybe via its putative downstream effector phosphatidylinositol-4-phosphate 5-kinase involved in membrane ruffling [36]. As mentioned above, RhoA and Rac seem to be involved in clathrin-dependent endocytosis, perhaps via interactions with synaptojanin 2 in the case of Rac [37]. In addition, RhoD regulates early endosome dynamic, perhaps by coordinating interactions with the cytoskeleton [38]. Similarly, RhoB is present on multivesicular bodies (MVBs) [39] and is involved in EGF receptor downregulation [40], suggestive of a signaling–trafficking cross-talk [28]. In yeast, it was recently shown that a lipid signaling pathway (based on sphingosine base synthesis) regulates a protein phosphorylation cascade required for internalization [41,42]. A potential target for this regulation pathway is the actin cytoskeleton, which plays an important role in yeast endocytosis. Whether mammalian cells also employ such a signaling pathway remains to be elucidated.

Evidence is now compelling that phosphoinositides are involved in the regulation of intracellular trafficking in addition to their role as second messenger, and these functions have previously been reviewed in detail [43–45]. These signaling lipids are now also implicated in receptor internalization and trafficking, and some directly bind components of the clathrin coat [46,47]. Since phosphoinositides are involved in signaling, trafficking and actin cytoskeleton remodeling, they obviously function as key regulators at the interface between these major cellular processes [32,46].

4. Clathrin-mediated endocytosis

Many proteins involved in the clathrin-mediated internalization process have now been characterized, including at the atomic level [48,49]. It is becoming clear that endocytosis is intimately coupled to signaling events at the cell surface via different networks of protein–protein interactions and post-translational modifications [50]. This presumably reflects the need for tight regulatory mechanisms between interactions occurring with the extracellular milieu and receptor trafficking.

In PC12 cells, activation of TrkA by the neurotrophin NGF was reported to increase clathrin-coated membrane formation and clathrin-mediated membrane trafficking [51]. In addition, recent studies showed that EGF binding to its receptor causes rapid phosphorylation of the clathrin heavy chain in a domain controlling clathrin assembly, via downstream activation of SRC kinase, indicating that receptor signaling contributes to regulate endocytosis via SRC [52]. EGF receptor endocytosis itself appears to depend on phospholipase D (PLD) [53], and PLD activity could be inhibited by accessory proteins of the brain clathrin coat, synaptojanin, AP180 and amphiphysin [54]. Below, we will briefly discuss individual components of the clathrin coat and accessory proteins.

The role of dynamin as a GTPase that plays an essential role in clathrin-coated vesicle formation is well documented [55,56], although its precise function is still debated [57]. Members of the dynamin family may also function on intracellular compartments [58], in addition to the caveolar pathway in endothelial cells [8], and perhaps phagocytosis [59]. Dynamin can interact with amphiphysin (see below) and syndapins, proteins which link endocytosis with the actin cyto-

skeleton [32]. Recent studies suggest that dynamin-2 may act as a signal-transducing GTPase, since its overexpression activates the transcription factor p53 and induces apoptosis [60]. Amphiphysin plays a key role in endocytosis by recruiting dynamin to clathrin-coated pits [61–63]. Amphiphysin I is found exclusively in brain and its phosphorylation inhibits binding to clathrin and the AP2 adaptor complex. Phosphorylation of dynamin and synaptojanin prevents binding to amphiphysin, indicating that phosphorylation regulates the cycle of the clathrin machinery [64,65]. Association of amphiphysin II with dynamin in PC12 cells was observed after stimulation with NGF, suggesting that the protein contributes to connect endocytosis and signal transduction pathways [66]. This dual function of amphiphysin II is also apparent in its reported ability to interact with the Ras exchange factor Sos in vitro [66].

Synaptojanin is an accessory protein of the clathrin machinery at the synapse. Clathrin-coated pits or vesicles accumulate at the nerve terminals of synaptojanin knock out mice [67], and in mutants of the synaptojanin orthologue in *Caenorhabditis elegans* (unc-26) [68]. The latter mutants also exhibit cytoskeletal abnormalities, and synaptojanin 2, a novel ubiquitously expressed isoform, was recently identified as a Rac1 effector [37]. Synaptojanin is a polyphosphoinositide phosphatase that binds directly to both $PI(4,5)P_2$ and proteins implicated in clathrin-mediated endocytosis [69,70]. These include amphiphysin, syndapins [71] and endophilin, a lysophosphatidic acid acyl transferase presumably involved in fission during clathrin-mediated synaptic vesicle endocytosis [72,73]. Synaptojanin may also interact with Grb2, an adaptor involved in mitogen-dependent signaling cascades [69].

Eps15 was originally identified as a substrate for the EGF receptor kinase [74] and subsequently implicated in endocytosis [75,76]. Eps15 and epsin are interacting partners for the α-adaptin subunit of the AP2 adaptor complex and triggered dephosphorylation increases binding to AP2 [77]. In addition, Eps15 phosphorylation was recently shown to be required for ligand-regulated, but not constitutive, endocytosis, suggesting that different mechanisms regulate trafficking of these receptors [78]. Epsin 1 was also shown to undergo nucleocytosolic shuttling and to interact with the transcription factor promyelocytic leukemia (PLZF), suggesting that epsin may be part of a signaling pathway involving endocytosis [79]. Intersectin, a multiple EH and SH3 domain-containing protein, is yet another protein that may link endocytosis with cell growth and differentiation [80,81]. It binds dynamin, Eps15 and epsin, and intersectin mutants inhibit transferrin receptor endocytosis [82,83]. Intersectin also appears to interact with the Ras exchange factor Sos, and overexpression of intersectin SH3 domain affects EGF-mediated mitogen-activated protein kinase (MAPK) activation through Ras [80].

5. The first endocytic station and the small GTPase Rab5

After internalization from most if not all entry routes, cell surface molecules, including receptors, are delivered to peripheral early endosomes. The small GTPase Rab5 is one of the key regulators of this early endocytic traffic, and it has been proposed that Rab5 functions as an organizer of early endosomal membranes via interactions with its effectors [84]. Evidence is now accumulating that signaling and Rab5-dependent pathways intersect in trafficking regulation.

Rab5, like Ras and other GTPases, cycles between GTP- and GDP-bound form, and GTP hydrolysis depends on a GTPase-activating protein (GAP). Several Rab5 GAPs have been identified. Some evidence, in fact, suggests that the catalytic domain of p120GAP, which acts on Ras and thereby regulates the EGF-dependent mitogenic response [85], interacts physically with Rab5 and stimulates its GTPase activity [86]. EGF stimulation was also reported to activate Rab5 functions, consistent with the notion that a relationship exists between receptor activation and internalization [87,88]. Another recently identified Rab5GAP, RN-tre, is regulated by the EGF receptor [89]. Interestingly, the Eps8 substrate of the EGF receptor seems to interact either with RN-tre in trafficking or with E3bl in signaling through Rac. It has also been proposed that PKB/akt, a protein kinase involved in insulin and PDGF signaling, mediates the stimulatory effect of Ras on endocytosis via Rab5 [90].

In addition to cycling between GTP- and GDP-bound states, Rab5, like other Rabs, cycles between the membrane and cytosol [84,91]. This latter cycle depends on GDI, which functions as a Rab vehicle in the aqueous environment of the cytosol. GDI is present in several isoforms, but clear functional differences have not been found amongst isoforms. Phosphorylation of GDI-2 on Tyr residues triggered by insulin stimulation of adipocytes enhances the selective extraction of the small GTPase Rab4, which is involved in receptor recycling to the cell surface [92–94]. Similarly, GDI-α is phosphorylated on Ser residues in vivo [95,96]. Recently we found that the MAPK p38 regulates the activity of GDI in the cytosolic cycle of Rab5, and that GDI Ser121 is essential in this process [97]. Selective activation of p38 MAPK increases endocytic rates in vivo, presumably allowing more efficient internalization of cell surface components for repair, storage, or degradation [97]. These observations emphasize the possibility that stress stimuli may contribute to the regulation of endocytosis and perhaps other pathways via p38 and GDI.

6. FYVE proteins

The early endosomal protein EEA1 [98] is also an effector of Rab5 essential for endosome fusion, and is recruited to endosomal membranes via binding not only to Rab5:GTP, but also to phosphatidylinositol-3-phosphate (PI(3)P) via a zinc finger referred to as FYVE domain [99–103]. These observations have led to the identification of a family of proteins bearing the consensus PI(3)P binding FYVE motif [104,105], and structural information has recently been obtained on PI(3)P–FYVE interactions [106,107]. Since PI(3)P may be present primarily at early steps of the endocytic pathway [108], one may wonder whether all FYVE proteins are preferentially targeted to these membranes.

The FYVE protein Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) localizes to early endosomes. Interactions with PI(3)P are required for localization and phosphorylation in response to EGF stimulation [109–111], but the role of the FYVE finger in localization is not clear [112]. Hrs is the homologue of the class E vps gene product Vps27p in yeast, a protein known to be required for vesicular trafficking [113]. Hrs was also found associated with STAM (signal-transducing adaptor molecule), perhaps counteracting STAM functions in cell growth signaling [114]. Targeted dis-

ruption of the Hrs gene in mice is embryonically lethal and the enlarged endosomes observed indicated that Hrs and endosomal function are required for ventral morphogenesis [111]. SARA (Smad anchor for receptor activation) is another FYVE protein that is presumably involved in TGFβ signaling, by interacting with Smads, which transmit signals from Ser/Thr kinase receptors to the nucleus [115].

Fab1p is a yeast protein essential for the maintenance of normal vacuolar morphology in yeast that contains an N-terminal FYVE domain, but also exhibits PI(3)P-5-OH kinase activity and thus converts PI(3)P into phosphatidylinositol-3,5-bisphosphate ($PI(3,5)P_2$) [105,116,117]. Fab1p was proposed to regulate vacuole membrane homeostasis, and perhaps anterograde transport to the vacuole [117], but also cargo-selective sorting into the vacuole lumen ([118] and see below). Interestingly, $PI(3,5)P_2$ was only recently discovered in mammalian cells, and is produced in response to osmotic stress [119,120]. PIKfyve, the putative human orthologue of Fab1p, contains an N-terminal FYVE domain and is localized to a peripheral punctate pattern [121,122], but its precise function has not been determined yet.

7. The recycling circuit

Once delivered to early endosomes, constitutively recycling receptors like the transferrin receptor are returned to the cell surface, at least in part via recycling endosomes, in contrast to downregulated receptors, which are transported to late endosomes and lysosomes [123]. Oxidative stress, which activates p38 MAPK, causes a rapid and specific redistribution of transferrin receptor from the cell surface to intracellular membranes, possibly recycling endosomes, presumably by interfering with receptor recycling [124,125]. Whether this mechanism depends on Rab GTPases involved in recycling via GDI phosphorylation by p38 MAPK [97] remains to be investigated. Rab11 is believed to regulate transport along this pathway [126]. Recent studies have identified Rip11 as a novel Rab11 effector in epithelial cells involved in apical membrane trafficking via recycling endosomes, and its membrane association appears to be regulated by phosphorylation [127].

The small GTPase Rab4 is also involved in the recycling pathway [92], perhaps at a step earlier than Rab11 [128]. Rab4 was proposed to regulate the insulin-dependent transporter GLUT-4 [129,130]. Conversely, insulin was shown to promote phosphorylation and activation of geranyl-geranyltransferase II in both 3T3 fibroblasts and adipocytes, resulting in increased amounts of geranyl-geranylated Rab3 and Rab4 [131]. Insulin was also shown to mediate the activation of farnesyl transferase and to increase the farnesylation of Ras, and the process was sensitive to MAPK inhibitors [132–134]. Similarly, Rab4 itself was reported to be phosphorylated by the insulin-activated extracellular signal-regulated kinase ERK1 [135], further involving the MAPK pathways in the control of membrane trafficking.

8. The pathway from early to late endosomes

In contrast to recycling receptors, some endocytosed proteins, including downregulated receptors, are efficiently transported from early to late endosomes [123]. While there is no doubt that sorting occurs at this step, little is known about the signals that may be involved. In mammalian cells, precise sequence motifs have only been identified in interleukin 2 receptor β-chain [136,137], *p*-selectin [138], and HIV Nef during CD4 downregulation [139]. Recent studies also point at the possible role of receptor ubiquitination in sorting at this step [140]. However, it is also becoming apparent that sorting in early endosomes may be lipid-based along both recycling and degradation pathways [46,141,142]. Indeed, lipids do not appear to be stochastically incorporated into either pathway, and they might thus contribute to sorting by facilitating preferential protein partitioning into defined lipidic environments. Transport from early to late endosomes is mediated by intermediates with a characteristic multivesicular appearance, referred to as MVBs or endosomal carrier vesicles (ECVs) [123], and their biogenesis is dependent on endosomal COPs and ARF1 in mammalian cells [143,144].

In yeast, evidence is accumulating that phosphoinositide signaling, via PI3K and PI(3)P, plays a critical role in MVB function [44]. In fact, $PI(3,5)P_2$ synthesis via the PI(3)P-5-OH kinase Fab1p is believed to be coupled to the regulation of MVB sorting [118]. Although the functional relationships between yeast and mammalian MVBs are still not completely clear, it has been reported that wortmannin, a drug that inhibits PI3K, inhibits MVB formation in animal cells [145]. PI(3)P was also found within internal membrane of ECV/MVBs but not of late endosomes [108]. Whether these signaling lipids promote membrane invagination remains to be clearly established. The yeast class E vps mutants all appear to interfere with MVB biogenesis [44,140]. Several other class E mutants have a mammalian homologue, Vps31p/alix, Vps23p/tsg101 and the recently identified vps4/SKD1. tsg101 is a tumor susceptibility gene 101 and its disruption induces cell transformation [146]. It was also proposed that tsg101 regulates lysosomal degradation of cell surface components including mitogenic receptors, perhaps contributing to the tumorigenic phenotype of the mutants [147,148].

In mammalian cells, ECV/MVBs, once formed on early endosomes, move towards late endosome on microtubules and then dock onto and fuse with late endosomes [123]. Little is known about the possible cross-talk between late endosomes/lysosomes and signaling pathways. However, recent studies identified a novel 14 kDa protein that interacts with the MAPK scaffold protein MP1 (MEK partner 1), on late endosomes/lysosomes [149], but its function on these membranes is unclear. In addition, major membrane remodeling occurs within late endosomes. Their invaginations selectively accumulate large amounts of the unusual phospholipid lysobisphosphatidic acid, which is involved in cholesterol and protein trafficking [150,151]. Both cholesterol and sphingolipids accumulate within late endosomes and within lysosomes in some lipid storage diseases, which may lead to signaling defects, since these raft lipids are presumably involved in signal transduction processes [152]. In addition, Rab prenylation and cholesterol synthesis use the same isoprene precursor. Hence diseases that affect cholesterol synthesis or storage may well affect Rab functions.

9. Conclusions and perspectives

Major progress has been made in unraveling the complex network of interactions between different signaling pathways and regulation of endocytic traffic. However, it is also clear that much work is still needed to dissect these networks at the

mechanistic level, including in development and in different tissues, and to characterize the precise role of each component. Signaling cascades appear to control not only internalization steps, but also the fate of internalized molecules along recycling and degradation pathways, by modulating trafficking routes. It is attractive to speculate that extracellular stimuli contribute to the endocytosis of cell surface components for repair, storage or degradation. In addition, traffic regulation by external stimuli emphasizes the possible role of this cross-talk in infection, aging and a number of degenerative diseases. Much effort has been invested in understanding the basic machineries regulating membrane traffic over the past decade. These have opened new research avenues which aim at a better understanding of the tuning mechanisms in membrane traffic.

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