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Ivan Dikic

Endosomes

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IVAN DIKIC received his M.D. and Ph.D. from the University of Zagreb, Croatia. After a postdoctoral tenure with Joseph Schlessinger at NYU, New York, USA, he became a group leader at the LICR, Uppsala, Sweden. In 2003, he was appointed a Professor of Biochemistry at the Goethe University Medical School, Frankfurt, Germany and from 2005 he co-ordinates work in the Tumor Biology Laboratory at the MedILS, Split, Croatia. His group focuses on the role of the E3 ligase Cbl and its associated proteins in signalling and receptor endocytosis and on the emerging role of ubiquitin and Ub-like modifiers as signalling devices controlling intracellular trafficking, gene transcription and DNA repair. He recently received 26th AACR Award for the Outstanding Achievement in Cancer Research.

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PREFACE

Endosomes are a heterogeneous population of endocytic vesicles and tubules that have captivated the interest of biologists for many years, partly due to their important cellular functions and partly due to their intriguing nature and dynamics. Endosomes represent a fascinating interconnected network of thousands of vesicles that transport various cargoes, mainly proteins and lipids, to distant cellular destinations. How endosomes function, what coordinates the molecular determinants at each step of their dynamic life cycle and what their biological and medical relevance is, are among the questions addressed in this book.

The past two decades have witnessed rapid strides in our understanding of the morphology and functions of endosomes (Chapter 1). In retrospect, the classical view of endocytic organelles has to give way to a more complex one, in that multiple, functionally distinct microdomains coexist within one endosomal structure. These microdomains are determined by a certain composition of distinct proteins or lipids that act as organizers of specific membrane domains (Chapters 2 and 3). Among the best-known facets of endosome function is their role in trafficking events at synapses, both in presynaptic and postsynaptic compartments of nerve cells (Chapter 4).

A detailed understanding of processes that regulate endosome fusion, clathrin-dependent receptor endocytosis and sorting to the recycling route or the degradative pathway is available via the integration of structural, molecular and biochemical studies on distinct endocytic adaptor proteins (Chapters 5, 6 and 7). Another important aspect is the processing of signals originating from internalized receptors and how their fate and signalling potency in cells are linked to their accumulation in distinct endosomal compartments, specifically during endocytosis of receptor tyrosine kinases (Chapters 8 and 9).

A topic of particular interest for the general public deals with the interface between endocytic trafficking and human diseases. Molecular views on aberrations in endosomes that are linked to the pathogenesis of various diseases are summarized (Chapter 10). The last two chapters are dedicated to the role of endosomes in viral entry and replication and how external pathogens and toxins hijack the endocytic machinery for their purposes by exploiting the cell's transport infrastructure (Chapters 11 and 12). Although quite similar in the general form of action many toxins appear to utilize different strategies to enter the cellular endosome system.

The concise format of the chapters and up-to-date molecular explanations of endosome functions may have a broad appeal for both students and scientists who wish to know more about the exciting world of trafficking in the cell.

Ivan Dikic

CHAPTER 1

Endosomes Come of Age

Ira Mellman*

> *"Endosomes are a population of endocytic vacuoles through which molecules internalized during endocytosis pass en route to lysosomes. In addition to this transport function, recent studies indicate that these organelles also act as clearing houses for incoming ligands, fluid components, and receptors."*
>
> —Helenius A et al. *Trends in Biochem Sci* 1983; 8:245-250.

In the late 1970s, a confluence of exciting observations triggered unprecedented interest in the functions and mechanisms of endocytosis. This activity, in turn, rapidly led to the identification of endosomes as a new and distinct organelle. Endosomes were first defined in 1983 by a simple statement that remains largely true even today (see above).[1] Endosomes were appreciated not only as intermediates on the pathway to lysosomes, but also on the pathway of receptor recycling where they were seen as being the primary site for the dissociation of ligand-receptor complexes and the return of unoccupied receptors back to the plasma membrane. In addition, for those receptors subject to "down regulation", endosomes were understood to be the place at which the crucial sorting decision was made between recycling and lysosomal transport. Many of these features were linked to the ability of endosomes to lower their internal pH via the activity of an ATP-dependent proton pump. Acidification was a key conceptual advance since it explained why many receptors discharged their ligands upon reaching endosomes and how incoming enveloped viruses fused with the endosomal membrane, initiating infection.

All this happened more than 21 years ago, meaning that even in Puritanical Western countries such as the United States, endosomes have (legally speaking) come of age. In other words, they can drink legally. This is a good thing since the primary activity mediated by endosomes is that of pinocytosis, literally "cell drinking".

Scientifically speaking, endosomes probably came of age long ago. Within a few years of their description, they became widely accepted as a new if incompletely understood organelle by cell biologists, with the concept rapidly spreading to allied fields. It is rare for a new organelle to be so quickly and broadly adopted, falling into the scientific lexicon so that endosomes now appear to have always existed (which, of course, they have). Yet, perhaps as a testament to their relative conceptual youth, our understanding of endosomes has continued develop at a remarkable pace. As will become abundantly clear in subsequent chapters, we have learned an immense amount about the mechanisms by which endosomes conduct their activities. Conversely, the study of endosomes has enabled the discovery of a wide array of basic principles in the broader field of molecular cell biology. This section will not attempt to review all of what we know concerning how endosomes work. It will, instead, take the opportunity to chart the development of how endosomes came to be understood, both functionally and mechanistically. The section is also written from the perspective of one who was privileged to be among

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those present at the beginning. Other perspectives exist, and others have contributed to the overall development of the field, even if they have not all been highlighted in this brief chapter. The goal is to place current advances in the context of the relatively brief history of endosomes as central players in cell biology.

The Discovery of Endosomes

To place our current understanding of endosomes in context, it is important to understand the scientific background to their discovery. Without attempting to provide a complete or systematic account, a few key conceptual highlights bear mentioning.

Two key observations were made in the mid- to late-1970s that not only launched the modern field of endocytosis, but also put in motion the events that would eventually lead to the identification of endosomes as distinct organelles. The first was from Steinman and colleagues, who used quantitative biochemical and EM approaches to demonstrate that mammalian cells in culture (fibroblasts, macrophages) internalized enormous areas of plasma membrane during constitutive endocytic activity, 50-200% every hour.[2,3] They reasoned that this rate of internalization was far greater than the capacity of cells for de novo membrane synthesis and concluded that the bulk of membrane must be recycled intact back to the plasma membrane. The second was from Brown, Goldstein and colleagues who were characterizing the receptor-mediated endocytosis of LDL. Highly influential, these studies demonstrated (among many other things) that more LDL particles were taken up and degraded than could be accounted for by new receptor synthesis, and that uptake was initiated at clathrin-coated pits.[4-6] In other words, internalized receptors were selectively internalized and rapidly recycled.

The principle of recycling during ligand uptake was rapidly extended to many other types of receptors. Moreover, our own early work demonstrated that, in general, a wide variety of membrane proteins were susceptible to internalization even during constitutive endocytosis.[7] In all cases, the internalized pools of membrane proteins remained predominantly long lived ($t_{1/2}$ ~24 hr), and could thus inferred (or in some cases shown directly) to escape intracellular degradation and recycle to the surface multiple times.[8]

There was a major conceptual problem posed by recycling, however. At the time, all endocytosis was viewed as having lysosomes as the primary intracellular destination. Indeed, even in the case of LDL receptor, bound LDL was seen as being released from its receptor after lysosomal delivery; the rapidity of transit through the degradative compartment presumably facilitating the receptor's escape. Since it appeared unlikely that receptors could survive repeated exposures the lysosomal proteases, increasing attention began to be paid towards a poorly described set of structures variably referred to as pinocytic vesicles, endocytic vesicles, pinosomes, receptosomes, CURL, or (perhaps most commonly) prelysosomal vacuoles. First hinted at in early cytochemical studies of endocytosis in the kidney by Strauss, these structures generally appeared more phase and electron lucent than did hydrolase-rich lysosomes.[1] It rapidly became clear, however, that they behaved as intermediates on the lysosomal pathway, accumulating internalized tracers (fluid phase components, receptor-bound ligands) transiently and prior to lysosomal arrival. Importantly, they also were relatively low with respect to their content of hydrolytic enzymes.

The first real indication that these prelysosomal structures were more than intermediates emanated from evidence in intact cells that they were acidic, and therefore might have an intrinsically important function. Previously, only lysosomes were recognized as acidic organelles in most cell types. The work of Helenius and colleagues was especially important in this regard. In the course of studying the entry and infection of enveloped animal viruses, they found that the low pH-induced fusion event required for entry occurred kinetically well before delivery of virions to hydrolase-rich lysosomes.[9] Work from Maxfield and from Klausner involved exposing cells to ligands coupled to the pH sensitive fluorochrome, showing (either by fluorescence microscopy or fluorometry) that the probes reached vesicles of acidic pH shortly after entry, presumably prior to lysosomal arrival.[10,11]

Our work made use of the recently established fact that prelysosomal vacuoles had a density on Percoll gradients that was much lower than lysosomes. This enabled us to physically separate lysosomes from endosomes in cells exposed to pH probes. In vitro, the low density endosomes were capable of ATP-dependent acidification, indicating that they, like lysosomes, contained an ATP-driven proton pump.[12] Mutant cells defective in virus entry (and killing by pH-activated bacterial toxins) in vivo exhibited a selective defect in endosomal acidification in vitro.[13] It is important to emphasize that the Percoll gradient experiments demonstrated that these prelysosomal structures were acidic but did not have the abundant hydrolytic activity found in lysosomes. In other words, endosomes were physically and functionally distinct from lysosomes.

So rapid was the progress that the published record was woefully unable to keep pace with the new ideas and experiments coming from many different laboratories. All involved seemed to understand that a new organelle was being born and, even more importantly, that a fundamental new pathway was being defined. Even while the acidification story was being developed, groups working on receptor-mediated endocytosis began accumulating evidence that the acidic pH in endosomes was responsible for the dissociation of many receptor-ligand complexes—and before their delivery to lysosomes. Favorite experiments at the time involved treating cells with acidophilic weak bases (chloroquine, ammonium chloride) or carboxylic ionophores (monensin, nigericin) that dissipated endosome (and lysosome) pH, showing that ligand discharge and sometimes receptor recycling could be blocked. Similarly, incubation of cells at intermediate temperatures (<20°C) appeared to block transit of all internalized substances to lysosomes, but allowed for continued ligand uptake and receptor recycling.

More direct evidence came from the work of Geuze and colleagues who used the emerging technique of immuno-gold EM on ultrathin cryosections (combined with some biochemistry) to define a prelysosomal compartment in which receptor and ligand physically dissociated.[14] The acid-dependent discharge of iron from internalized transferrin (Tfn) was shown, in Percoll gradients, to occur in low density endosome-containing fractions.[10] Interestingly, Tfn itself was never found to transfer to high density fractions, suggesting that it recycled to and from endosomes without encountering lysosomes. Much the same was found for the FcγRII-B2 IgG receptor, which was shown to recycle from endosomes while monovalent, but transferred from endosomes to lysosomes when cross-linked.[15,16]

Such experiments provided powerful functional evidence that endosomes existed, and that they played an essential role in receptor recycling. As in biochemistry and murder investigations, however, unless an actual protein—or "body"—can be purified or produced, the existence of endosomes as a discrete compartment would remain in doubt. A great effort was thus expended attempting to isolate these structures. Cell fractionation is difficult under the best of circumstances, but a particular challenge was presented by endosomes, an organelle with no known intrinsic markers, with no obvious morphological features, and that was not particularly abundant. We were fortunate to obtain perhaps the most enriched endosome populations, using an electrophoretic technique that separated membranes on the basis of inherent net charge.[17,18] The resulting membrane fractions had a predictable composition, similar to but distinct from the plasma membrane and lysosomes. Endosomes exist.

Taking all this together, the view emerged that endosomes provided a distinct intracellular site at which internalized receptor-ligand complexes were first dissociated and then sorted to distinct destinations: receptors to the plasma membrane for reuse and discharged ligands to lysosomes for degradation.[1] Figure 1 presents a very early diagram of the pathway, which turned out to be fairly prescient in several respects.

The ability specify different fates for membrane and luminal components was remarkable, and further defined the ability of endosomes to sort the container from the fluid it contained. From both EM and quantitative measurements, it was concluded that a good fraction of this sorting activity could reflect simple Euclidean considerations. If endocytosis was mediated by spherical clathrin coated vesicles while recycling was initiated by the long tubular extensions

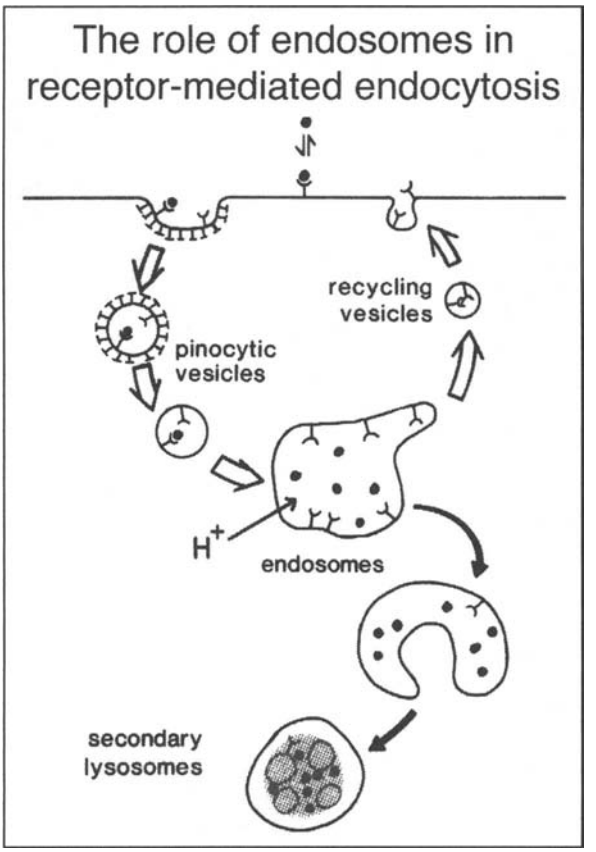

Figure 1. An early diagram of the role of endosomes in receptor-mediated endocytosis, circa 1983. Drawn by Ari Helenius, Ira Mellman, and Margaret Moench.

characteristic of endosomes, the surface to volume ratio of the recycling vehicles would be far higher than that of the incoming vesicles. Thus, recycling would favor the return of membrane over fluid, leading to the intracellular accumulation of any macromolecule no longer bound to a receptor on the endosomal membrane.[1,19] Adaptor-mediated specificity could clearly be superimposed on this underlying mechanism (see below).

Endosome Subpopulations

From the earliest stages, it was suspected that endosomes did not comprise a simple, homogeneous population but rather a collection of compartments with distinct properties and functions. Early indications came from morphological analyses: endosomes labeled after a brief (1-2 min) uptake of endocytic tracers were generally found in the cell periphery, but then appeared in more centrally located, often multivesicular structures prior to arrival in hydrolase-rich lysosomes. Further, certain recycling receptors, notably Tfn receptor, were in many cell types found to be concentrated in the perinuclear area, close to the Golgi complex and microtubule organizing center.[20,21] Unlike the peripheral compartment, these structures labeled at relatively long times after endocytosis.

The advent of molecular markers for distinct organelle subsets—lgp/lamp, mannose-6-phosphate receptors (MPR), Rab proteins, SNAREs—helped clarify the functions and

interrelationships of these various endosomal populations. From cell fractionation experiments, several important concepts were clarified, while others created. We found, for example, that it was possible to physically separate two distinct endosome populations that labeled early (1-2 min) or late (10-15 min) after the uptake of fluid-phase markers. Designated early and late endosomes, both populations were found to be acidic (although late endosomes were more acidic) but otherwise distinct in composition: early endosomes contained recycling receptors and little in the way of lysosomal markers (lgp/lamp, MPR) while late endosomes had the opposite phenotype.[18] Detailed immunofluorescence and immuno-EM experiments soon established definitions for each compartment: early endosomes (MPR and lgp/lamp-negative, recycling receptor-positive), late endosomes (MPR and lgp/lamp-positive), and lysosomes (MPR-negative, lgp/lamp-positive).[22,23] As Rab proteins began to be described, these too were incorporated into the functional definition of endosome compartments, although they were often found in more than one species of endosome (e.g., Rab4/5, early endosomes; Rab7/9, late endosomes).[24-26]

Of special interest was Rab11 (recently joined by Rab8 and others) as marking that subpopulation of Tfn receptor-containing endosomes near the MTOC.[27,28] With this observation, these "recycling endosomes" took on the significance as a third endosome compartment, as opposed to a simple population of transport vesicles. Recycling endosomes always appeared distinct from early endosomes in that they generally did not contain detectable amounts of dissociated ligands or fluid phase markers in transit to lysosomes.[21] Kinetic analysis revealed another difference: although recycling endosomes contained recycling receptors, the recycling endosome pool took far longer to return to the plasma membrane (20-30 min) than the pool that reached only early endosomes (3-4 min).[20,29]

Recycling endosomes became viewed as containing an intracellular pool of recycling membrane components that can be pressed into service when needed, such as possibly providing extra membrane for particle uptake during phagocytosis[30] or possibly during directed cell migration. These intriguing structures may also have essential roles in signal transduction, regulation, and secretion. Their proximity to the Golgi complex continues to cause great confusion as to whether a given marker or event is localized to the Golgi or to endosomes. Indeed, the distinction between recycling endosomes and terminal Golgi elements such as the trans-Golgi network (TGN) may be more semantic than instructive, as will be discussed below.

Endosome Maturation

One of the popular controversies during the first 15 years of endosome research was the issue of endosome biogenesis. Where do they come from? Although the problem has not been entirely solved, the topic appears to have achieved a quiet equilibrium. Helenius et al[1] mused that there were two possible scenarios (Fig. 2). In the maturation model, endosomes might gradually be transformed into lysosomes by virtue of reciprocal fusion events with other organelles and transport vesicles, coupled with the selective recycling of receptors. In the vesicle shuttle model, endosomes (or their subpopulations) were viewed as stable organelles whose contents passed between them via distinct transport vesicles, much as has been understood for transport across the Golgi stack. The model was included not because we felt there was any particular evidence for it in the case of endosomes, but rather due to the strong influence of prevailing views of the Golgi complex. As an aside, it is amusing to recall the impatience expressed by colleagues studying the Golgi who wondered why the endosome community was unable to distinguish between these two models. How the tables have turned!

Considering the considerable capacity for endosomes to move in the cytoplasm, as evidenced from the earliest time lapse video microscopy, it always seemed likely that endosomes were highly dynamic structures that "matured" one into the next. Indeed, direct fusions of endosomes with lysosomes could be visualized.[8] A variety of other more direct considerations now also support a view more in accord with gradual maturation, at least on the lysosomal pathway. For example, the accumulation of multivesicular inclusions pathognomonic of late

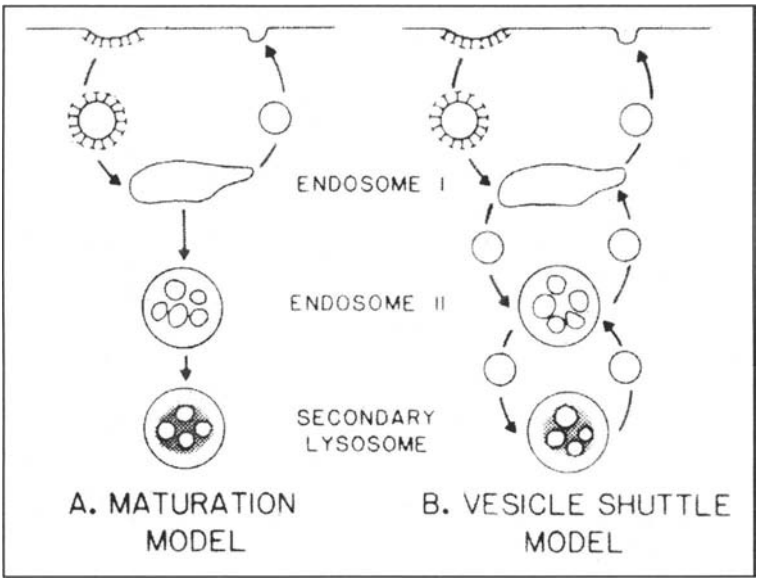

Figure 2. Two views of endosome biogenesis: maturation vs. vesicle shuttle.Reprinted from reference 1, ©1983, with permission from Elsevier.

endosomes and lysosomes begins with the functional assembly of ESCRT complexes at the surface of early endosomes.[31] Combined with the concerted addition of new lysosomal components,[32] the gradual accumulation of multivesicular inclusions emphasizes a remodeling process that converts early endosomes into late endosomes. That the entire endosomal system could turn over rapidly was demonstrated by the nearly complete (and reversible) loss of endosomes in cells where clathrin-mediated endocytosis was arrested by expression of a dynamin GTPase mutant.[33] Thus, endosomes cannot be considered as preexisting stable structures if their presence depends on continuous membrane input by endocytosis. Combined with the prolific amounts of membrane known to move through the system each hour,[8] it is almost a semantic impossibility to consider the endosomal apparatus as being anything other than subject to dynamic remodeling, i.e., maturation.

At the same time, it must also be true that at least some specific transport events take place. The removal of receptors for delivery to the plasma membrane or recycling endosomes must reflect the formation of transport vehicles (vesicles or tubules). Similarly, the return of MPR to the Golgi complex where it must reside in order to capture newly synthesized lysosomal enzymes must also involve a selective recapture pathway.[26] Much the same can be said for the selective return of TGN proteins (TGN38, furin) from endosomes to the Golgi complex.[34,35]

From such considerations, our current view has emerged of endosomes as highly dynamic structures that are closely interrelated and that at least to some degree "mature" from early endosomes to late endosomes to lysosomes. Certainly, some selective sorting or vesicle formation events are also likely to occur, but these do not seem to be stable structures that communicate with each other via a system of small, transport vesicles.

Acidification

It is obvious that a key feature of endosomes is their acidic pH. In general, the farther one proceeds towards lysosomes, the lower the pH. Thus, depending on the pH dissociation profile of a given receptor-ligand complex or fusion threshold of a given enveloped virus, dissociation

or infection will occur in different endosomal compartments. Early endosomes are generally given as having a pH of 6-6.8, late endosomes 5-6, and lysosomes 4.5-5, although these numbers probably vary considerably in different cell types.[36]

As mentioned above, it was established early on that endosomes (and lysosomes) contained a proton ATPase. Based on the inhibitor profile of the pump,[12] it was predicted that it would be a member of a unique class of ATPase dedicated to the acidification of both endocytic and secretory organelles. Indeed, subsequent work revealed that the class of "vacuolar ATPase" (V-ATPase) was unique, but was nevertheless closely related to the large F1-F0-like proton ATPase of acidophilic bacteria.[37] Consisting of the same general organization as all such pumps (a soluble multisubunit V1 sector containing the ATPase portion, a membrane-associated multisubunit V0 sector, containing the proton pore), different cells and possibly even different organelles contain different combinatorial forms of the ATPase. The functional significance of such heterogeneity is unclear.

Why early endosomes have a less acidic pH than late endosomes is still not known for certain, but several factors no doubt contribute. The number of pumps may be a factor, with their highest enrichment potentially being in lysosomes. Ion permeabilities of organelle membranes is also different, and is quite likely to contribute to equilibrium pH (Fig. 3). The V-ATPase is electrogenic, meaning that inward pumping of protons is accompanied by the accumulation of an interior-positive membrane potential which, importantly, impedes proton transport probably by directly inhibiting the pump. Endosomal membranes are less leaky to other anions (e.g., Cl^-) and alkalai cations (K^+, Na^+) whose movement across membranes dissipates the forming electrical potential, thus limiting the chemical gradient of protons that the V-ATPase can achieve.[38] The endosome membrane is also quite leaky to protons, making acidification dependent on a dynamic flux of protons per unit time, rather than on a specific number of protons translocated per endosome. Other electrogenic pumps (e.g., Na-K-ATPase, not shown in the diagram) may further limit the pH attained in early endosomes by contributing to the interior-positive potential; the Na-K-ATPase translocates 3 Na^+ in for every 2 K^+ out.[39] Pump assembly (i.e., formation of functional V1-V0 complexes) or subunit composition may also contribute to pH heterogeneity.[40]

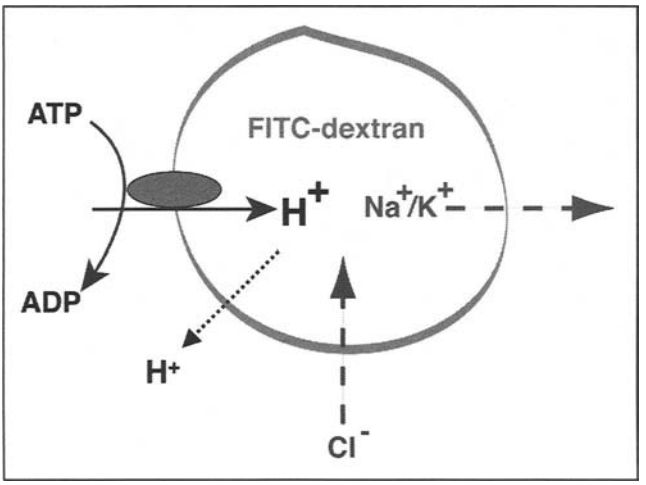

Figure 3. The mechanism of ATP-dependent endosome acidification. Protons are transported into the endosome lumen by the V-ATPase, with their equilibrium concentration determined by the electrochemical gradient reflecting the influx of anions, efflux of alkalai cations, and rate of proton leak back to the cytosol.

Despite the importance of acidification to membrane traffic on the endocytic pathway, the V-ATPase has remained under-studied, perhaps due to its complexity. Given recent results suggesting that its subunits may be directly or indirectly involved in vesicle fusion,[41] and that its assembly can be regulated by certain signaling pathways,[40] further attention would appear warranted.

Sorting Stations at the Crossroads of Membrane Traffic

A primary function of endosomes is molecular sorting, with different endosome compartments playing different roles. Not only are a variety of endocytic sorting events hosted by endosomes, but there are also clear examples where the biosynthetic pathway makes use of endosomal sorting capacity.

Sorting Membrane and Content

The first and perhaps most important sorting function during endocytosis is the separation of membrane from contents, an activity that appears to be the purview of early endosomes. Without this function, cells would not be able to retain or concentrate internalized macromolecules. Early endosomes host both the dissociation of ligands from most rapidly recycling receptors (e.g., LDL receptor) and the physical sorting of the dissociated ligands and other contents in the endosome lumen from membrane and receptors intended for recycling. This is why early endosomes are occasionally referred to as "sorting endosomes" (a term we avoid since all endosomes sort in one way or another). As evidence for sorting of membrane and contents in early endosomes, one need only look at recycling endosomes: it is rare for them to contain fluid-dissolved macromolecules.[21,36] Some fluid does appear to recycle from early endosomes to the extracellular milieu, but this occurs rapidly, and quite possibly reflects the rapid route of recycling that avoids transit through recycling endosomes. Indeed, quantitative measurements have indicated that only 25% of each cohort of internalized receptors reaches recycling endosomes (in MDCK or CHO cells).[29] In any event, it has long been clear, based on quantitative EM measurements, that the concentration of internalized solutes increases as one moves towards later compartments.[8] Thus, endosomes must also sort fluid from solutes by allowing water to egress across the endosomal membrane.

Down Regulation

Since the early work of Cohen,[42] it was apparent that inclusion of receptors in forming MVB's was associated with receptor down regulation.[43] By being removed from the endosome's limiting membrane, the receptors are effectively converted into endosomal content and as such can be taken to lysosomes. Great strides have been made in understanding the biochemistry and genetics of these events both in yeast and animal cells.[44] As will be considered elsewhere in this volume, it is now understood that receptors are typically marked for MVB inclusion by mono-ubiquitination (often following receptor activation following ligand binding or cross-linking). This modification is recognized by proteins such as Hrs (in animal cells) that then trigger recruitment of the ESCRT complex. These complexes initiate the invagination of the endosome's limiting membrane, sequestering the ubiquitin-marked receptors. Since MVBs are classified morphologically as late endosomes, it was presumed that late endosomes were the primary site for this sorting event. While that may be true, as mentioned earlier ESCRT complex proteins, as well as proteins involved in recognizing the mono-ubiquitination signals associated with receptor down regulation first bind to early endosomes.[31] Thus, early endosomes may be progressively converted into late endosomes by concerted MVB formation.

Lysosomal Biogenesis

It has long been known that MPR's carry newly synthesized lysosomal enzymes from the TGN to early and/or late endosomes. Upon delivery, the acidic pH facilitates dissociation of the receptor-ligand complex resulting in the delivery of the discharged enzymes to lysosomes, along with any internalized content.[32] By making use of a selective targeting event initiated at

the TGN, the cell basically makes use of endosomal sorting to serve a specific function on the secretory pathway.

MHC Class II Molecules and Antigen Presentation

Another example of an intersection between the biosynthetic and endocytic pathways can be found in antigen presenting cells of the immune system.[45] Here, newly synthesized MHC class II molecules, in association with their targeting chaperone "invariant chain", are targeted from the TGN to endosomes (early or late?), where the complexes can exhibit one of two fates. If the invariant chain is cleaved by endosomal-lysosomal proteases (typically cathepsin S), the released $\alpha\beta$ dimers can proceed (via recycling) to the plasma membrane. If not, then the entire complex is transferred to lysosomes, likely following inclusion on forming MVB's.[46] This pathway is an essential aspect of the immune response. It is in endocytic compartments that newly synthesized MHC class II molecules encounter exogenous antigens internalized by endocytosis, and thus acquire the 10-15-mer peptides that are required for presentation to T lymphocytes. The ability to mount immunity to foreign antigens and tolerance to self antigens is therefore intimately dependent on the sorting functions of endosomes, a point illustrated dramatically in dendritic cells, the immune systems most important antigen presenting cell.[47]

Endosomal Sorting in Polarized Cells

Endosomal sorting and recycling in polarized epithelial cell presents an additional challenge: cells must maintain the ability to selectively return incoming receptors to the basolateral or apical surfaces. Interestingly, endosomes accomplish this task by recognizing the same cytoplasmic and luminal sorting signals on basolateral and apical proteins as are recognized in the secretory pathway.[48,49] Thus, endosomes must be capable of signal-dependent sorting, and not just the bulk separation of recycling receptors from endosomal contents or lysosomally-directed components.

Given the equivalence of signals used, it is possible that polarized sorting in endosomes uses the same sets of adaptors as polarized sorting on the secretory pathway. In the case of tyrosine-based basolateral targeting signals, one complex may be the AP-1B clathrin adaptor.[50] At present, the best evidence supports the possibility that signal-dependent sorting occurs uniquely in recycling endosomes (Fig. 4). Although not yet conclusive, kinetic analysis suggests that signals control sorting only in the slowly recycling pool of receptors such as transferrin receptor, apparently at the level of recycling endosomes in MDCK cells.[29] Moreover, during transcytosis, a common pool of recycling endosomes may receive input from both the apical and basolateral domains,[29] although there is some immunofluorescence evidence suggesting an additional, specialized endosomal compartment that fulfills this role.[51]

More recently, we have found that many of the components which in polarized cells are essential for basolateral transport on the secretory pathway actually localize to recycling endosomes in MDCK cells.[28,52] Indeed, both immuno-EM and functional evidence indicates that recycling endosomes serve as an intermediate on the biosynthetic pathway to the surface.[53] Thus, there may be a common site for all polarized sorting in epithelial cells, and that site would represent yet another remarkable convergence of the endocytic and secretory pathways.

Endosomes in Specialized Cells

The example of polarized epithelial cell raises the issue of whether specialized cell types exhibit endosome specializations that serve cell type-specific functions. Although unresolved, there is considerable evidence that this may be the case. In adipocytes, for example, vesicles containing the Glut4 glucose transporter may form by a specific budding event from an endosomal intermediate.[54,55] Much the same idea has been proposed in the case of synaptic vesicle formation in neuroendocrine cells.[56] In such cases, one can imagine that all of the components required for the formation of specialized vesicles are delivered by endocytosis to endosomes, at which site they are sorted and sequestered into populations of recycling vesicles whose recruitment to the plasma membrane can be carefully regulated.

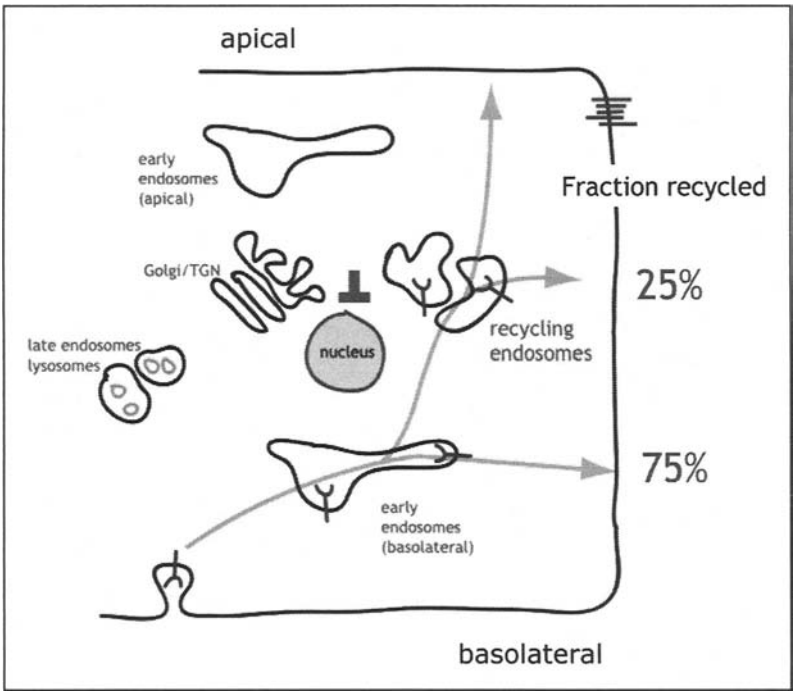

Figure 4. Proposed scheme of sorting by recycling endosomes in polarized cells. As in non-polarized cells, only ~25% of each internalized cohort of receptors actually reaches the perinuclear recycling endosomes. Here, they are interrogated for the presence of basolateral targeting signals by adaptor complexes such as AP-1B. If a productive interaction occurs, these receptors are efficiently recycled back to the basolateral surface. If not, they are transported from recycling endosomes to the apical surface, thereby exhibiting "transcytosis". Recycling traffic from early endosomes in the basolateral cytoplasm is thought to be largely to the basolateral surface, but is signal-independent: transport occurs regardless of whether a specific basolateral targeting signal is present. See reference 29.

In antigen presenting cells, identifying the actual site(s) at which antigen is degraded and loaded onto MHC class II molecules has long been a problem of interest. Initial work characterized a late endosome-like compartment (MIIC) which was thought to represent an organelle specialized for peptide loading, but are now thought simply to reflect "conventional" multivesicular late endosomes that happen to be loaded with MHC class II and associated molecules.[57-59] Whether, in B cells, these structures represent the major loading site, however, remains unclear.

With the demonstration that dendritic cells play by far the most important role in initiating antigen-specific immune responses, attention has recently been shifted to the endocytic system in this cell type. Although unremarkable at first glance, dendritic cells possess the ability to completely reorganize their endosomes and lysosomes in response to stimuli that activate their antigen processing and presentation abilities.[47] Targeting of MHC class II is altered, lysosomal acidification is activated due to V-ATPase assembly, and peptide-MHC class II complexes are induced to form. Strikingly, the late endosomal/lysosomal structures that contain the bulk of MHC class II molecules in resting "immature" cells tubulates, and transfers its MHC class II to the plasma membrane.[59-62] This rearrangement involves the loss of MHC class II-positive internal vesicles from these MVB-like structures, raising the possibility that they somehow have fused with the endosome's limiting membrane (or were degraded upon activation of dendritic cell protease activity that also occurs upon maturation).[40] If the internal vesicles did indeed

fuse, this would be a totally unexpected fate for a class of membranes until now thought to be marked for degradation.

Conclusions: Endosomes in Cell Biology

In this brief review, we have tried to place our burgeoning understanding of endosomes in the context of their discovery and initial characterization, not so very long ago. We have explored no more than a few of the most striking functional attributes and molecular insights that have emerged. For example, one key function pertains to the role of endosomes in signal transduction. The surface expression of signaling receptors can be rapidly modulated by regulating traffic to and from the recycling endosome pool. These same structures may also serve as platforms for generating signals. Similarly, in the TGFβ pathway, critical adaptor molecules are found only in early endosomes, meaning that signaling cannot occur until the receptor is internalized.[63,64]

The efforts of a great many groups spanning two decades has established endosomes as key and important organelles in a wide range of cell types and pathways. This work has contributed to our understanding of fundamental cell biology in other ways, however. Endosomes have provided a remarkable convenient platform on which to develop concepts concerning the function of Rab proteins, effectors, SNAREs and tethers in processes ranging from the formation of membrane microdomains to membrane fusion.[65] Arguably, more has been learned in this respect from the study of endosomes than from any other organelle.

Endosomes may have come of age quickly, but they have done it well.

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CHAPTER 2

Lipid Membrane Domains in Endosomes

Julien Chevallier and Jean Gruenberg*

Abstract

It has long been appreciated that the membranes of endosomes contain different regions or domains visible by electron microscopy, including, for example, the intraluminal and limiting membranes of multivesicular compartments. Evidence also shows that endosomes contain different lipid territories, and that such territories overlap with morphologically visible domains. Here, we will discuss recent advances in our understanding of the role of these specialized membrane domains and protein-lipid assemblies in the endocytic pathway leading to lysosomes.

Introduction

Eukaryotic cells need to be in constant communication with their environment in order to perform most of their functions, such as the transmission of neuronal, metabolic, and proliferative signals, the uptake of nutrients, or to protect the organism from microbial invasion, to name only a few. During endocytosis, cell surface receptors and their ligands, as well as particles or solutes present in the extracellular space, are taken up by vesicles that form at the plasma membrane, sorted to early endosomes, and then targeted to various intracellular destinations (Fig. 1). As a consequence, the lumen of endosomes—and of all organelles of the vacuolar apparatus—is topologically equivalent with the extracellular space. Lysosomes are a common final destination for endocytosed macromolecules, where digestive enzymes degrade them. The resulting metabolites are then released into the cytoplasm where they can be recycled by incorporation into newly synthesised macromolecules.

Endosomes (like biosynthetic organelles) exhibit a wide variety of shapes and structures, which can be easily visualised by classical electron microscopy. They range from the clusters of thin, long tubules of recycling endosomes to late endosomes that contain onion-like sheets of internal membranes, tubules or vesicles (multivesicular or multilamellar endosomes). While little is known about the molecular mechanisms controlling organelle shape and biogenesis, or the functional significance of such diversity, evidence shows that organelles in the endocytic pathway are composed of a mosaic of structural and functional regions.[1,2] These regions consist, at least in part, of specialized protein–lipid domains within the plane of the membrane or of protein complexes associated to specific membrane lipids. Indeed, many cytosolic proteins interact with membranes by binding not only to proteins but also to lipids, often through multiple protein-lipid and protein-protein interactions.[3-5] Such interactions are not easily studied, however, and it should be emphasized that physiologically-relevant parameters, e.g., kinetic constants, are not always known. In any case, the dynamic interplay between such specialized

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protein–lipid domains may provide a driving force responsible both for the specific organization of each compartment and for the movement of cargo molecules.

Lipids provide the physical support of organelle membranes, acting as a barrier for water-soluble molecules and as a solvent for the hydrophobic domains of membrane proteins. By contributing to the intrinsic properties of membranes, such as thickness, asymmetry, and curvature, lipids can potentially regulate protein movement and distribution.[6,7] Evidence is accumulating that some short- and long-lived lipids have a restricted distribution in the plane of the bilayer, thereby forming transient or more stable microdomains.[8] In particular, cholesterol and sphingolipids were proposed to form a separate liquid-ordered phase in the liquid-disordered matrix of the lipid bilayer (lipid rafts), thereby functioning as platforms that can incorporate distinct classes of proteins, and thus regulate numerous cellular processes, including signalling, sorting and infection.[9-11] Here, we will discuss the organization of endosomes into different membrane domains, and, in particular, evidence supporting the notion that, in animal cells, endosomes along the degradation pathway leading to lysosomes contain more than one type of membrane domains with different lipid compositions and functions.

Mophologically-Visible Domains

As mentionned above, it has long been appreciated that endosomes contain specialized membrane regions or domains that are visible by electron microscopy. While thin tubules form the elements of recycling endosomes, all endosomes along the degradation pathway leading to lysosomes can accumulate internal membranes in their lumen, thus appearing multivesicular or multilamellar. At early stage of the latter degradation pathway in animal cells, multivesicular endosomes form regularly-shaped and large vesicles (diameter $\approx 0.4 - 0.5$ µm) with densely packed intraluminal membranes that appear like small vesicles or tubules (diameter: 50-80 nm).[12] Such multivesicular endosomes form on early endosomal membranes and mediate transport to late endosomes, and have thus been referred to as endosomal carrier vesicles (ECVs) or multivesicular bodies (MVBs) according to their function or appearance, respectively.

By contrast with ECV/MVBs, the large vesicular elements of late endosomes are often less regularly shaped, with sizes ranging from ≈ 0.5 to 1.0 µm, and can exhibit a more intraluminal organization, including internal vesicles or tubules (like ECV/MVBs), onion-like sheets (multilamellar endosomes), or a mixture of both. In some cells, late endosomes also accumulate electron-lucent materials, perhaps of lipidic origin that can form elongated, needle-like structures (e.g., BHK cells and AtT20 cells). Clearly, this bird's eye view of these organelles reveals that, beyond all mechanistic debates, endosomes along the degradation pathway contain different morphologically-visible membrane regions or domains.

Lipid Distribution

All lipids do not behave as bulk constituents of the bilayer and are not all stochastically distributed within membranes of endocytic organelles.[4,8,13] In particular, studies with toxins that bind cell surface glycolipids,[14] or with fluorescent lipid analogs[8,15,16] indicate that different lipids or lipid analogs inserted into the plasma membrane may follow different intracellular routes after endocytosis. In addition, evidence is accumulating that endosomal membranes also contain different lipids at successive stage of the degradation pathway.

Over the past few years, phosphoinositides have emerged as key-regulators of membrane traffic by controlling the localisation and/or activity of effector proteins, through the action of kinases and phosphatases that mediate highly localised changes in the level of phosphoinositides, providing a means for the temporal and spatial regulation of effectors.[3,7] In the endocytic pathway, phosphatidylinositol 4,5-bisphosphate, $PI(4,5)P_2$, plays a crucial role during internalization, by recruiting proteins implicated in endocytosis, including the AP-2 adaptor, the GTPase dynamin, and proteins that contain an ENTH (Epsin NH2-Terminal Homology)-like domain, e.g., CALM (clathrin assembly lymphoid myeloid leukemia protein), AP180 and Epsin.[3] In addition to $PI(4,5)P_2$, phosphatidylinositol 3-phosphate, PI(3)P, also regulates

endocytic membrane traffic, but presumably at the next step of the pathway, on early endosomes. PI(3)P is generated at least in part on early endosomal membranes via the recruitment of the PI3K hVPS34 by the active GTP-bound Rab5, and thus contributes to the formation of Rab5 effector platforms.[2] PI(3)P plays a major role in endocytic traffic through interactions with the FYVE zinc finger domain that is present in over 10 different proteins, including Rab5 effectors, with a wide range of structures and functions in mammalian cells. In addition, the human genome also contains many (≈ 50) genes that encode proteins with the phosphoinositide-binding Phox homology (PX) domain. Amongst those that have been characterized, many PX-proteins bind PI(3)P, in particular some members of the sorting nexin family.[17,18] Interestingly, labelling of cryo-sections with a tandem-FYVE construct revealed that PI(3)P is abundant in the internal membranes of ECV/MVBs, and, to a much lesser extent, of late endosomes.[19] PI(3)P also serves as a substrate for the PtdIns3P 5-kinase Fab1/PIKfyve that generates PtdIns(3,5)P_2. While Fab1/PIKfyve and its product PtdIns(3,5)P_2 clearly play a crucial role in protein sorting,[20-24] the precise localization of the lipid in endosomal membrane is not known.

A very similar distribution was observed for cholesterol, when probing cryo-sections with a derivative of the cholesterol-binding Theta-toxin (perfringolysin O),[25] perhaps suggesting that intra-endosomal cholesterol and PI3P are both abundant within the same ECV/MVB internal membranes (see Fig 1, green membranes). But, it is also possible that the two lipids distribute preferentially within different pools of internal vesicles. By contrast, late endosomes accumulate large amounts (>15 Mol%) of the unconventional phospholipids lyso-bisphosphatidic acid (LBPA) or bis-monoacylglycerophosphate (BMP), and this lipid is not detected elsewhere in the cell.[26] Immunogold labelling of cryosections with anti-LBPA antibodies shows that the lipid is abundant in internal membranes,[26] where it does not seem to colocalize with PI3P[19] or cholesterol[25] (see Fig 1, red membranes). Altogether these studies indicate that endosomes along the degradation pathway in mammalian cells may contain at least two types of intraluminal membranes with different lipid compositions, including perhaps some enriched in PI3P-cholesterol and LBPA, respectively (Fig. 1, outline).

It should be emphasized that it has not been possible until now to correlate the different morphologically visible regions of late endosome internal membranes (e.g., multivesicular vs. multilamellar) with differences in biophysical or biochemical properties. However, the sub-organellar fractionation of late endosomes revealed not only that internal membranes could be separated on gradients without detergent from the limiting membrane of the organelle, but also that at least two populations of internal membranes with a different lipid composition can be separated from each other.[27] In addition, late endosomes also contain detergent-resistant membranes (DRMs) enriched in glycosylphosphatidyl inositol (GPI)-anchored proteins and cholesterol, presumably rafts.[28] Recent studies, in fact, suggest that two populations of DRMs with a different protein composition are present in late endosomes, within internal membranes and at the limiting membrane, respectively.[29] Whether intraluminal DRMs correspond to the cholesterol-rich internal membranes visible by electron microscopy[25] is not known. But, these studies indicate that (at least) two populations of internal membranes continue to coexist in late endosomes, further supporting the notions discussed above (see Fig 1).

Functionally Different Membrane Domains

In addition to these differences in morphology and composition, evidence is also accumulating that endosomes in mammalian cells contain more than one population of functionally different membrane domains. Indeed, when some signalling receptors are downregulated in the presence of excess ligand, they are endocytosed and then rapidly appear within the intraluminal vesicles of endosomes, thus providing an efficient means to turn off signalling, by removing the receptor from its interacting signalling partners present in the cytosol.[30,31] Major progress has been made in understanding the molecular mechanisms that control this sorting event, which allows the selective incorporation of receptors destined to be degraded within these intraluminal membrane invaginations. Some downregulated receptors are ubiquitinylated and this modification

is responsible for sorting into forming ECV/MVBs through binding to Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate), which also binds PI(3)P via its FYVE domain, and ESCRTs-I, -II and -III (endosomal sorting complexes required for transport).[32-34] Conversely, Hrs and ESCRT complexes are believed to drive membrane invagination itself, since the process is inhibited in yeast and Drosophila mutants with impaired Hrs (VPS28 in yeast) functions, and in mammalian cells treated with Hrs siRNAs.[33,35,36] This mechanism, which is conserved from yeast to mammals, leads to the notion that internal vesicles with their cargo of lipids, downregulated receptors and, presumably, other proteins are transported to lysosomes for degradation. Indeed, intraluminal vesicles accumulate in the vacuole of yeast degradation mutants.[33]

However, intraluminal membranes present in the late endosomes of animal cells also contain proteins and lipids that are not destined for the lysosomes. LBPA, which accumulates within intraluminal membranes, is in fact poorly degradable, perhaps because of its unconventional stereochemistry.[37] Late endosome internal membranes also typically contain members of the tetraspanin family including CD63/Lamp3,[38] which are presumably not destined to be degraded. Moreover, MHC (major histocompatibility complex) class II molecules are predominantly found within internal membranes of late endosomes (MIICs) in dendritic cells. Upon cell activation, these molecules are rapidly transported to the cell surface demonstrating that back-transport from late endosomes internal membranes can occur, at least in these cells, presumably via tubules,[39] that may form at the expense of internal membranes via back-fusion.[12] In addition, the mannose-6-phosphate receptor (MPR), which delivers newly-synthesized lysosomal enzymes to endosomes and lysosomes, cycles between the trans-Golgi network (TGN) and endosomes, with the bulk present in the TGN at steady state in some cell types. While in transit in endosomes, MPR is found within late endosome internal membranes,[40] where it accumulates in cells containing endocytosed antibodies against LBPA.[26] The situation may be different in yeast cells. It is not clear whether yeast cells contain LBPA, and cargoes that recycle from intraluminal vesicles to the limiting membrane of yeast endosomes have not been identified. Even if a related recycling pathway may exist in yeast, it is likely to be of lesser importance than in animal cells. Indeed, although MVEs have been observed in yeast,[41] membrane invaginations and internal vesicles seem to be far more abundant in the endosomes of animal cells, and are readily visible at a steady state. It thus appears that, in addition to the downregulation pathway conserved from yeast to man, animal cells have evolved a more elaborate membrane system in late endosomes for more efficient reutilization and sorting of specialized lipid and protein (see outline, Fig. 1).

Endosomes in animal cells thus seem to contain at least two morphologically, biochemically and functionally different populations of intraluminal vesicles (Fig. 1). It is tempting thus to speculate that the internal vesicles that form on early endosmal membranes via a mechanism involving the short-lived lipid PI3P and the PI3P-binding protein Hrs, as well as ESCRT complexes, correspond to the vesicles that accumulate in ECV/MVBs and contain both cholesterol and PI3P, as well as cargo molecules that need to be degraded, in particular signalling receptors. These vesicles contained in the endosomal lumen, are then presumably transported via late endosomes to the lysosomes for complete degradation. In addition, late endosomes also seem to contain a second population of internal vesicles, which are rich in LBPA, and thus poorly degradable. These membranes contain proteins that are not destined for the lysosomes, but can be returned to the limiting membrane (presumably via back-fusion of the intraluminal vesicles) and then transported to other cellular destinations.

LBPA and Alix/ALP1

While the biophysical and biochemical properties of internal membranes are still poorly understood, progress has been made in understanding some of the properties and functions of late endosome internal membranes rich in LBPA. This lipid is presumably synthesized in situ within the acidic organelles of the endocytic pathway,[42] and, in BHK cells, is predominantly ($\approx$ 90%) present as the 2,2'-dioleoyl isoform[27,43] (Fig. 2). But, the β-position of the glycerol

backbone, to which the oleoyl chains are esterified in 2,2'-LBPA, is thermodynamically unstable, and fatty acids can migrate to the α-positions, thus forming 3,3'-LBPA.[43] Such acyl chain migration may well contribute to regulate the function of the lipid in vivo: 2,2-LBPA, but presumably not 3,3'-LBPA, is predicted to be cone shaped,[27,43] and may thus facilitate the formation of membrane invaginations. Indeed, 2,2'-LBPA, but not 3,3'-LBPA, drives the spontaneous formation of multivesicular liposomes, when the liposome lumen is acidified to the pH (≈ 5.5) of late endosomes.[44] Hence, 2,2'-LBPA, the major late endosomal isoform, is endowed with the intrinsic capacity to stimulate internal vesicle formation within acidic liposomes, and thus to generate structures that resemble late endosomes where the lipid is found in vivo.[44] This mechanism is attractive. Invagination occurs towards the endosomal lumen, and is thus unlikely to depend on cytosolic machineries (e.g., coat proteins) that control vesicle formation in the topologically opposite direction.

This invagination process is likely to be regulated by proteins in vivo, and indeed it was found to depend on Alix/ALP1, which, in turn, binds liposomes containing 2,2'-LBPA, but not 3,3'-LBPA.[44] Moreover, Alix downregulation with siRNAs affect both late endosome membrane organization and the cellular LBPA content.[44] Although the precise function of Alix remains to be unravelled, other studies already provide some insights into its biological role.[5] Alix, which was identified as a partner of ALG-2 involved in apoptosis, interacts with proteins that play a role in signalling and endocytosis,[5] and is the mammalian homologue of yeast Bro1p/Vps31p, which regulates MVB formation in concert with ESCRT proteins. Consistently, Alix together with ESCRT proteins play a role in HIV budding at the plasma membrane, presumably reflecting the capacity of the virus to hijack proteins that normally drive the topologically equivalent process of invagination within endosomes.[45,46] Whether Bro1p/Vps31p functions are LBPA-dependent in yeast is not known, since LBPA was not detected in yeast. However, it is possible that Alix acquired the capacity to interact with LBPA later in evolution, since Alix and Bro1p are relatively distantly related (≈ 24% identity by the Jotun Hein method, and 17% by the clustal method).

Intraluminal Traffic

Several lines of evidence indicate that LBPA and Alix play a direct role in the dynamics of late endosome internal membranes in vivo. Endocytosed anti-LBPA antibodies interfere with protein and lipid sorting and trafficking, membrane transport and motility at the level of late endosomes, and these defects phenocopy the cholesterol storage disorder Niemann-Pick type C or NPC.[47-49] Presumably, antibodies, by binding their antigen within the endosomal lumen,[26] inhibit the dynamic properties of this intraluminal membrane system, and thus prevent the movement of proteins and lipids from intraluminal vesicles to the limiting membrane. Consistently, cholesterol accumulation within late endosomes, including in NPC cells, recapitulates the same defects as observed with anti-LBPA antibodies,[1] presumably because excess cholesterol, beyond the endosomal capacity, also collapses the dynamics of internal membrane and causes an endosomal traffic jam.[50] These observations lead to the notion that intraluminal proteins and lipids can be delivered to the limiting membrane of the organelle, by back-fusion of intraluminal vesicles with the limiting membrane, similarly to the transport of MHC class II molecules in antigen-presenting cells[12]—a process inhibited by anti-LBPA antibodies and sensitive to excess cholesterol.

Further evidence supporting this notion comes from studies with anthrax toxin and with the enveloped virus vesicular stomatitis virus (VSV). The protective antigen (PA) of anthrax toxin binds to a cell surface receptor, undergoes heptamerization, and then recruits the enzymatic subunits, the lethal factor (LF) and the edema factor (EF). After endocytosis of the complex, and membrane insertion of PA, LF and EF are ultimately delivered to the cytoplasm where their targets reside. Recent studies show that membrane insertion of PA already occurs in early endosomes, possibly only in the multivesicular regions, but that subsequent delivery of LF to the cytoplasm occurs preferentially later in the endocytic pathway, relies on the dynamics

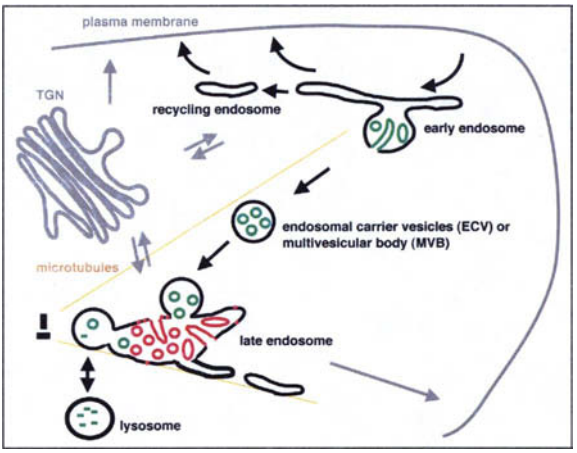

Figure 1. See page 20 for figure legend.

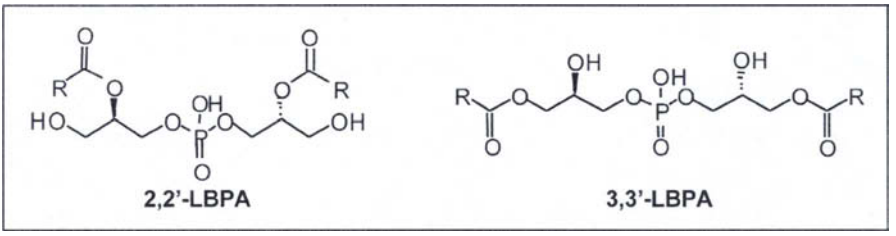

Figure 2. See page 20 for figure legend.

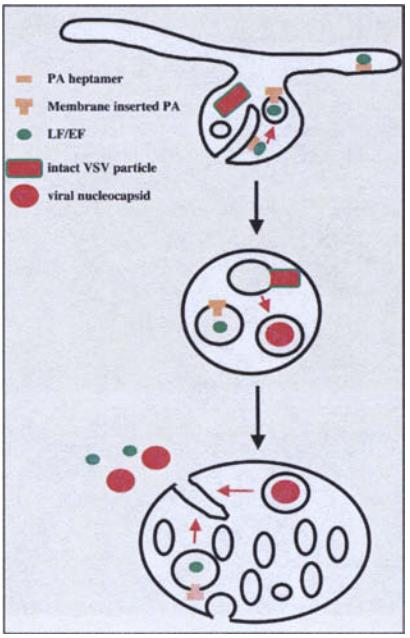

Figure 3. See page 20 for figure legend.

Figure 1. Endosomes and internal membranes. The figure shows the outline of the endocytic pathway in animal cells, as well as recycling routes to the trans-Golgi network or TGN (followed for example by MPR molecules), and to the plasma membrane (e.g., MHC class II in antigen-presenting cells). The two types of intraluminal membranes discussed in the text are indicated: (1) Green membranes contain down-regulated signalling receptors and may be enriched in PI3P and perhaps cholesterol. These vesicles and their cargoes are presumably transport to lysosomes for degradation. (2) Red membranes contain LBPA and may regulate the dynamics of late endosomes internal membranes via fission from and fusion with the limiting membrane. Much like at other transport steps in the cell,[53] two views have been proposed to account for endosome biogenesis, through their formation from a stable early endosome or through the maturation of early endosomes. These two alternative models have already been discussed extensively elsewhere, and there is still no experimental evidence that proves or discloses either model. It is not easy to discriminate between a multivesicular endosome that forms after the detachment of recycling tubules and one that forms as a result of direct detachment from a "stable" early endosome.

Figure 2. Structure of LBPA. The chemical composition of 2,2'-LBPA and 3,3'-LBPA is shown. In the BHK cells, the major (≈ 90%) isoform is 2,2'-dioleoyl-LBPA (R is C18:1). LBPA esterified at the 2 and 2' of the glycerol backbone is predicted to be cone-shaped and has the capacity to deform bilayers,[44] consistently with the role of lipid shapes in generating membrane curvature.[6]

Figure 3. Dynamics of intraluminal membranes. The figure outlines the intra-endosomal routes of anthrax toxin and VSV. After internalization, the protective antigen (PA) with bound lethal factor and edema factor (EF) inserts into the membrane of internal vesicles, presumably in early endosomes, and thereby allows the translocation of the LF and EF into the lumen of internal vesicles. These are transported to late endosomes. LF and EF are then released into the cytoplasm via fusion of internal vesicles with the limiting membrane. Similarly, after endocytosis, intact VSV particles appear in early endosomes. These virions then fuse with intra-endosomal vesicles presumably within ECV/MVBs (see Fig. 1). Internal vesicles are transported to late endosomes, where the nucleocapsids are then released into the cytoplasm via fusion of internal vesicles with the limiting membrane.

of internal vesicles of multivesicular late endosomes, and, in particular, is inhibited by Alix downexpression with siRNAs[51] (Fig. 3). Similarly, after VSV endocytosis and beyond early endosomes, the low endosomal pH triggers the fusion of the viral envelope with endosomal membranes, releasing the viral nucleocapsid into the cytosol, where replication of the viral genome occurs. Much like intoxication with anthrax, VSV infection is inhibited by Alix siRNAs[44] (Fig. 2). Consistently, recent studies, indicate that viral fusion already occurs in transport intermediates between early and late endosomes, presumably releasing the nucleocapsid within the lumen of intra-endosomal vesicles, where it remains hidden. Transport to late endosomes is then required for the nucleocapsid to be delivered to the cytoplasm, in a process that depends on LBPA and Alix.[52] Hence, it thus seems that VSV and anthrax toxin have hijacked the same mechanism for infection and intoxication, via the back-fusion of intraluminal vesicles with the late endosome limiting membrane, to overcome the diffusion barrier imposed by the cortical actin cytoskeleton, and reach more efficiently the perinuclear region of the cell (Fig. 3).

Conclusions

The molecular events that regulate fission and fusion within the endosomal lumen clearly remain to be elucidated. It is far from clear how fission and fusion can be controlled by cytosolic machineries from the opposite side of the membrane—as opposed to the role of coat proteins and SNAREs in intracellular transport. Within late endosomes, our previously published data argue that these intraluminal fission and fusion events may depend, at least in part, on the intrinsic properties of the bilayer itself, via LBPA.[27,44] LBPA-rich membranes may have a high propensity to interact spontaneously with the limiting bilayer, involving some sort of kiss-and-run fission and fusion events. However, proteins are likely to control the process, since, in particular, fission may remain frustrated if uncontrolled fusion occurs concomitantly, and vice-versa. Our

previous data suggested that Alix negatively controls the invagination process by binding LBPA-rich membranes,[44] and in turn, Alix is likely to act in concert with other proteins, including ESCRTs.[5] Thus, a simple and naïve view is that Alix traps the membrane intermediate in fission or fusion, by interacting with LBPA as the lipid appears on the limiting bilayer, and thereby controls the rates of both vesicle formation (invagination) and consumption (fusion). This view is attractive, because it provides a reasonable mechanistic explanation for the coupling, which must exist between invagination and back-fusion. Indeed, if uncoupled, the internal membrane pool would disappear (uncontrolled back–fusion), or the organelle collapse (uncontrolled fission).

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CHAPTER 3

Rab Domains on Endosomes

Marta Miaczynska* and Marino Zerial

Abstract

Small GTPases of the Rab family have been long recognized to be key regulators of membrane trafficking. However, recent studies have uncovered their more fundamental role as determinants of organelle biogenesis and maintenance in all cells. Rab proteins acting in the endocytic pathway were shown to occupy nonoverlapping, morphologically and biochemically distinct domains on membranes of endosomes. Molecular characterization of Rab5 and its effectors revealed basic principles by which this GTPase mediates local changes in membrane structure and function, thus organizing a specific domain on early endosomes. Rab domains on endosomes appear to coordinate multiple functions related to membrane trafficking, organelle motility and signal transduction and are dynamically linked through the activity of bivalent Rab effectors. The concept of Rab proteins acting as membrane organizers provides a framework explaining the biogenesis of endocytic organelles composed of separate but functionally coupled domains which are arranged in a dynamic fashion.

Introduction

Eukaryotic cells are characterized by highly compartmentalized structure comprising numerous membrane-bound organelles, which ensure a precise spatial segregation and temporal control of various physiological processes. Throughout evolution, polarization of cells and their functional specialization into tissues have been accompanied by changes in their intracellular organization, often resulting in specialized organelles present only in certain cell types, such as apical and basolateral endosomes in epithelial cells, melanosomes in pigment cells or dense-core granules in various secretory cells. The overall morphology and function of intracellular compartments have been investigated intensively for a few decades. However, studies of the sub-structure and the organization of membranes limiting the intracellular compartments have become possible only more recently owing to the developments of experimental techniques. A general concept emerging from studies at the sub-organellar level reveals that components constituting a membrane of a compartment, both proteins and lipids, are not stochastically distributed but rather segregated and concentrated in distinct but dynamic domains within the plane of the membrane. Importantly, this further implies that various functions within an organelle can be efficiently compartmentalized and assigned to appropriate domains. Finally, the identity of an organelle will be therefore determined by a particular combination of functional domains, which ensure a spatio-temporal regulation of processes taking place within this organelle.

Various mechanisms, based on protein-protein, protein-lipid and lipid-lipid interactions, appear to be responsible for formation of membrane domains. A classical example of local accumulation of specific proteins and lipids is the formation of coat complexes, required for

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concentration of selected cargo molecules and the budding of transport vesicles.[1] Coat components ensure generation of curved membrane pits, sorting and incorporation of cargo into them and finally their scission from the membrane. This series of events is orchestrated by an intricate network of interactions between proteins and lipids, occurring in a spatially confined membrane domain with a strict temporal control. Among various lipid classes involved, phosphoinositides seem to play a particularly important role in recruiting cytosolic proteins to the membrane.[2] Interestingly, also certain proteins seem to have a peculiar ability to drive the formation of specialized membrane structures, such as caveolin or von Willebrand factor which, again through further interactions with other proteins and lipids, direct biogenesis of caveolae[3] or Weibel-Palade bodies,[4] respectively. Interactions between lipids can also underlie a nonhomogenous distribution of membrane components, as exemplified by lipid rafts. Lipid rafts are generated through interactions of sphingolipids and cholesterol and selectively incorporate certain transmembrane proteins, fulfilling important regulatory functions.[5] In case of all membrane domains, local concentration of components, kept in place by mutual interactions, is crucial for confining specific functions to certain membrane regions.

This chapter will be devoted to another group of proteins involved in organelle biogenesis and postulated to form membrane domains that are small GTPases of the Rab family. In particular, we will discuss the mechanisms by which Rab proteins orchestrate intracellular transport via the spatio-temporal regulation of effector proteins that assemble into biochemically distinct and functionally specialized membrane domains on endosomal organelles.

Rab Proteins As Determinants of Organelle Identity

The Rab family of proteins comprises over 60 members (designated Ypt proteins in yeast) which regulate virtually all membrane trafficking steps within the secretory and endocytic pathways. They coordinate subsequent stages of transport such as formation of vesicles, their motility along cytoskeletal filaments and finally their docking and fusion with target membranes (reviewed in refs. 6,7). Newly synthesized, GDP-bound Rab proteins form a cytosolic complex with a Rab escort protein (REP) which presents them to geranylgeranyl transferase II for prenylation and subsequently delivers the modified proteins to their target membranes.[8,9] Following this initial, REP-mediated membrane targeting event, the activity of Rab proteins is regulated by two overlapping cycles (reviewed in refs. 10,11). First, Rab proteins locked in an inactive, GDP-bound form can shuttle between the cytosol and specific target membranes, chaperoned by Rab GDP dissociation inhibitor (GDI).[12] GDI is structurally related to REP; however it cannot mediate the prenylation of Rab proteins.[13] Second, once delivered to the membrane via a GDI displacement factor (GDF),[14] Rab proteins undergo cycles of activation resulting from binding of GTP, followed by inactivation via GTP hydrolysis. The nucleotide cycle of each Rab protein is catalyzed by specific GDP/GTP-exchange factors (GEFs) and GTPase activating proteins (GAPs). Active Rab proteins present on the membrane interact with their specific effectors, mediating downstream processes such as budding, motility or fusion of vesicles. Due to the regulated cycles of GTP binding and hydrolysis, followed by binding of effectors, Rab proteins ensure temporal and spatial control of membrane transport.[15]

Each Rab protein is characterized by a specific and restricted intracellular distribution. Several Rabs have been localized to endosomal compartments and/or implicated in the regulation of various endocytic events. At present, this list includes: Rab4,[16] Rab5,[17] Rab7,[18] Rab9,[19] Rab11,[20] Rab13,[21] Rab14,[22] Rab15,[23] Rab17,[24,25] Rab18,[26] Rab20,[26] Rab21,[27] Rab22,[28] Rab23,[29] Rab25,[30] Rab34[31] and Rab39.[32] However, only a few of these proteins have been characterized in more detail. Among them, the ubiquitously expressed Rab4, Rab11 and Rab15 are present on early and recycling endosomes,[20,23,33-35] although a Golgi-associated pool of Rab11 also exists.[36] Rab5 localizes to clathrin-coated vesicles and early endosomes,[17] Rab22 is present on early endosomes,[28,37] while Rab7 and Rab9 are distributed to late endosomes.[18,19] Some Rab proteins such as Rab17,18,20 or 25 are specifically expressed in

epithelial cells where Rab17 and 25 appear to regulate polarized endocytosis through the apical recycling compartment.[25,30]

Due to their specific localization to various membrane compartments throughout the cell,[6] Rab proteins have been long recognized as organelle markers and their role as rate-limiting regulators of transport is well established.[15,17,38,39] However, more recent data using knockout/knockdown approaches argue that Rab proteins are not merely "compartment tags" but play an active role in the biogenesis of membrane organelles, being one of the key determinants of compartment identity. Two studies have recently provided strong experimental evidence in support of this concept. In *Drosophila*, zygotic loss of Rab5 causes drastic disruption of endosomes during initial stages of development and, eventually, leads to embryonic lethality.[40] Knockdown of Rab9 by RNA interference (RNAi) in cultured mammalian cells decreased the overall size of late endosomes.[41] Strikingly, it also reduced a number of particular subclasses of these endosomes, such as multilamellar and dense-tubule–containing late endosomes/lysosomes, but not multivesicular endosomes. These data strongly argue that Rab proteins play a crucial role in the biogenesis of endocytic organelles.

Rab Proteins As Organizers of Membrane Domains

Are Rab proteins evenly distributed throughout the organelle membrane? Some initial observations indicated that Rab5 was not present uniformly on the membrane of early endosomes but rather concentrated in clusters.[42,43] Such assemblies were visualized by light microscopy on endosomes enlarged due to the overexpression of an activated mutant of Rab5 (Rab5Q79L). Interestingly, Rab5-enriched clusters contained also a Rab5 effector EEA1[42] and were concentrated in regions mediating fusion between endosomes.[43] Similarly, docking of yeast vacuoles before fusion appears to involve formation of "vertex" ring-shaped microdomains around the periphery of the apposed membranes. These vertices are selectively enriched in Rab GTPase Ypt7p together with its effector complex Vps class C/HOPS.[44] Thus, Rab proteins with the associated effector proteins appear to mark particular regions of the organelle membrane, thus predestining them for certain functions.

However, most organelles appear to contain more then one Rab protein, raising questions about the distribution and any functional relationships between Rab proteins within the same membrane compartment. A systematic analysis of distribution of endosomal Rab5, Rab4 and Rab11 with respect to endocytic cargo (transferrin) has been conducted by quantitative light microscopy analysis.[34] Strikingly, the analyzed Rab proteins exhibited a largely nonoverlapping distribution, with each protein occupying distinct, often adjacent membrane regions within the individual endosomal compartments. While early endosomes appeared to be composed of domains containing Rab5 and Rab4, recycling endosomes represented a mosaic of Rab4 and Rab11 domains (Fig. 1). Such distribution was nonstochastic, as Rab5 was present in various amounts in different pools: about 50% of all Rab5 structures did not contain Rab4 or Rab11, 30% of them colocalized only with Rab4 and 20% contained both Rab4 and Rab11. In contrast, 30% of Rab4 compartments were positive for Rab5; other 30% contained Rab11, 20% colocalized with both Rab5 and Rab11, while the remaining 20% were Rab5- and Rab11-negative. Moreover, internalized transferrin (endocytic cargo destined for recycling) colocalized sequentially first with Rab5 domains, then with Rab4- and finally with Rab11-enriched regions. Similar functional segregation of Rab4 and Rab11 domains with respect to cargo has been demonstrated for recycling of glycosphingolipids using fluorescent analogue of lactosylceramide as a marker,[45] underscoring the notion that lipids and proteins segregate into different membrane (micro)domains. Further analyses revealed that domains containing distinct Rab proteins exhibited different pharmacological properties.[34,45] While domains on Rab4 and Rab11 endosomes appeared to be sensitive to brefeldin A (BFA), Rab5 domains were resistant to BFA-induced tubulation but instead affected by phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin. These data indicated that other components of endosomal membranes are also selectively concentrated in specific Rab domains.

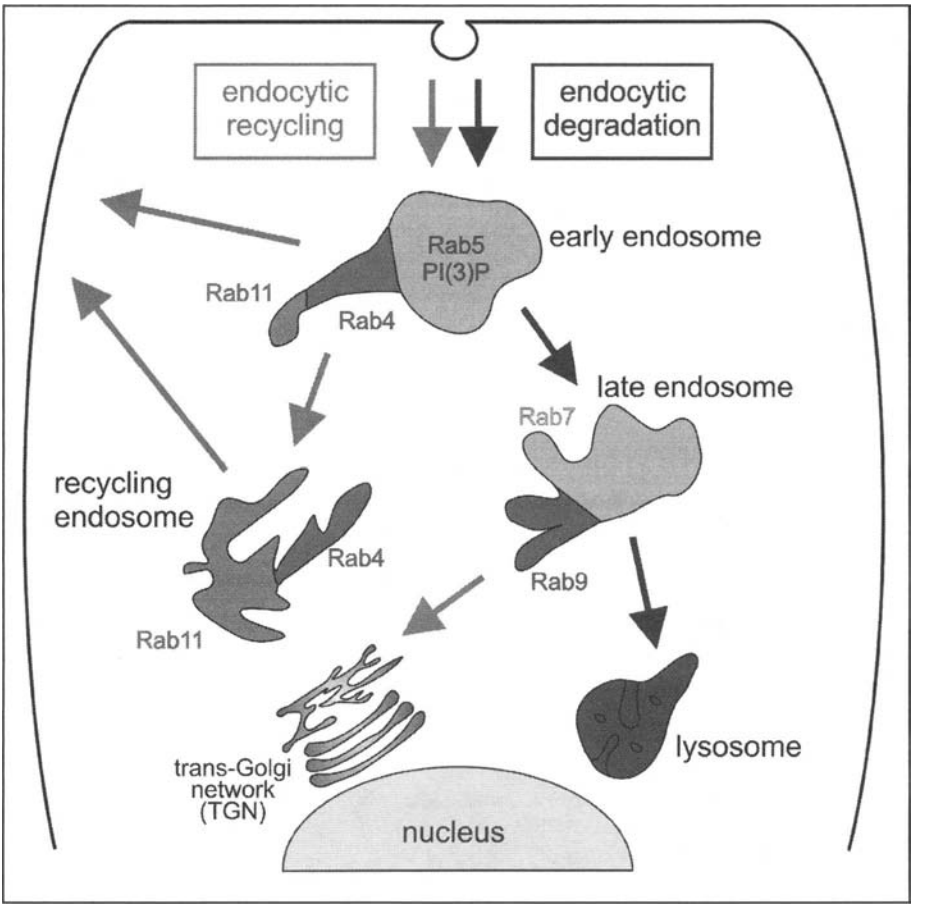

Figure 1. Distribution of Rab domains along the endocytic pathways. Only major endosomal compartments are shown. Cargo destined for degradation or recycling enters the early endosome via the Rab5 domain. Subsequently, cargo is sorted either for recycling via Rab4 and Rab11 domains or for degradation via Rab7 domains. Rab9 domains on late endosomes direct transport of certain cargo (e.g., CI-MPR) towards the trans-Golgi network.

A complementary study[46] demonstrated a similar principle of organization of late endosomes where Rab7 and Rab9 were shown to occupy distinct domains within a single organelle. Rab9 domains, regulating transport between late endosomes and trans-Golgi network (TGN), are enriched in specific cargo (cation-independent mannose 6-phosphate receptors, CI-MPR) and contain Rab9 effector TIP47. In contrast, Rab7 domains are postulated to mediate the transport of cargo from early endosomes towards degradation in lysosomes. Thus, two different trafficking routes through late endosomes appear to be spatially separated and regulated by distinct Rab domains. Overall, the endosomal compartments can therefore be considered as a mosaic of various domains occupied by Rab proteins and their effectors.

Molecular Assembly of a Rab Domain

A key feature of Rab proteins acting as organizers of membrane domains is their ability to mediate local changes in membrane structure and function. This is achieved via a series of interactions with a large number of protein effectors, among them lipid-modifying enzymes.

The latter can affect the lipid composition of the bilayer by local generation and accumulation of particular lipid species. In parallel, Rab-mediated recruitment of cytosolic proteins can locally modulate the protein content of the membrane, creating a microenvironment enriched in certain molecules and thus predestined for certain functions. Cooperativity of effector recruitment, membrane anchoring of effectors through binding to specific lipids and lateral interactions between recruited effectors constitute the major principles of domain formation and maintenance by Rab proteins. These principles are illustrated by the best-studied example of a domain coordinated by Rab5 on the membrane of early endosomes (Fig. 2).

Rab5, initially delivered to the endosomal membrane in an inactive, GDP-bound form, undergoes activation catalyzed by specific GEFs, such as Rabex-5,[47] RIN1,[48] RIN2[49] or RIN3.[50] Interestingly, Rabex-5 is stably complexed to a Rab5 effector Rabaptin-5.[47] Such physical association of a GEF and an effector ensures a synergistic action of both molecules.[51] On the one side, Rabaptin-5 increases the exchange activity of Rabex-5 on Rab5. On the other side, Rab5-dependent recruitment of Rabaptin-5 to early endosomes is completely dependent on its physical association with Rabex-5. Rab5 on early endosomes undergoes continuous cycles of nucleotide binding and hydrolysis,[15] the latter process assisted by specific GAP proteins such as RN-tre[52] or RabGAP-5.[53] Active Rab5 can further interact with other effectors, one of them being the type III PI3K complex hVPS34/p150.[54] Although this complex is targeted to early endosomes Rab5-independently,[55] most likely via lipid-modified p150,[56] an interaction between Rab5-GTP and p150 occurring on the membrane is believed to locally activate the PI3K and thus restrict the production of phosphatidylinositol 3-phosphate (PI(3)P) to a particular domain. The resulting accumulation of Rab5-GTP and PI(3)P creates high-affinity binding sites for recruitment of cytosolic effectors such as EEA1,[57] Rabenosyn-5[58] or Rabankyrin-5[59] able to bind both PI(3)P via a FYVE motif (named after Fab1p, YOTB, Vac1p and EEA1[60]) and Rab5 (Fig. 2). Additionally, lateral interactions between the recruited effectors and other membrane components, such as the elements of the SNARE [soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor] machinery, lead to formation of large oligomers,[42] stabilizing the molecular backbone of the domain. Indeed, experiments using fluorescence recovery after photobleaching (FRAP) on individual Rab5Q79L-enlarged endosomes demonstrated a restricted lateral mobility of GFP-Rab5Q79L molecules, consistent with the existence of oligomeric effector complexes on the early endosome membrane.[61] Recruitment of membrane tethering/fusion complexes is further coupled to the cytoskeletal transport machinery. The plus-end kinesin KIF16B is recruited to early endosomes Rab5- and PI(3)P-dependently and is rate-limiting for the association and movement of early endosomes with microtubules [62] (see below).

In addition to its interaction with hVPS34/p150, the type III PI3K, on the early endosomes, Rab5 binds to and stimulates the activity of p110β/p85α, the type I PI3K which is recruited to the plasma membrane in response to growth factor or cytokine stimulation and Ras activation [54,63,64] (Fig. 2). This type of PI3K converts PI(4,5)P_2 and PI(4)P to PI(3,4,5)$_3$ and PI(3,4)P_2, respectively, which in turn play an important role in growth factor signaling, actin rearrangements, phagocytosis and cell motility by recruiting appropriate effector proteins to the plasma membrane.[63] Indeed, Rab5 has been previously implicated in the regulation of cell motility, [65] phagocytosis [66-68] and various aspects of growth factor signaling [48,69-71] (see below). Surprisingly, Rab5 appears to coordinate not only the production of the 3-phosphorylated inositides but also their turnover through interactions with the specific phosphatases.[64] Rab5 binds directly and stimulates the activity of the type II inositol 5-phosphatase and the type I α PI(3,4)P_2 4-phosphatase, thus promoting the gradual dephosphorylation of PI(3,4,5)$_3$ generated at the plasma membrane to PI(3)P accumulating on early endosomes (Fig. 2). These recent data provided first evidence for a Rab protein regulating both generation and turnover of phosphoinositides through an enzymatic cascade of effectors.

Other Rab proteins also appear to be functionally linked to different lipid-modifying enzymes, even though the molecular mechanisms of such interactions have not been explored

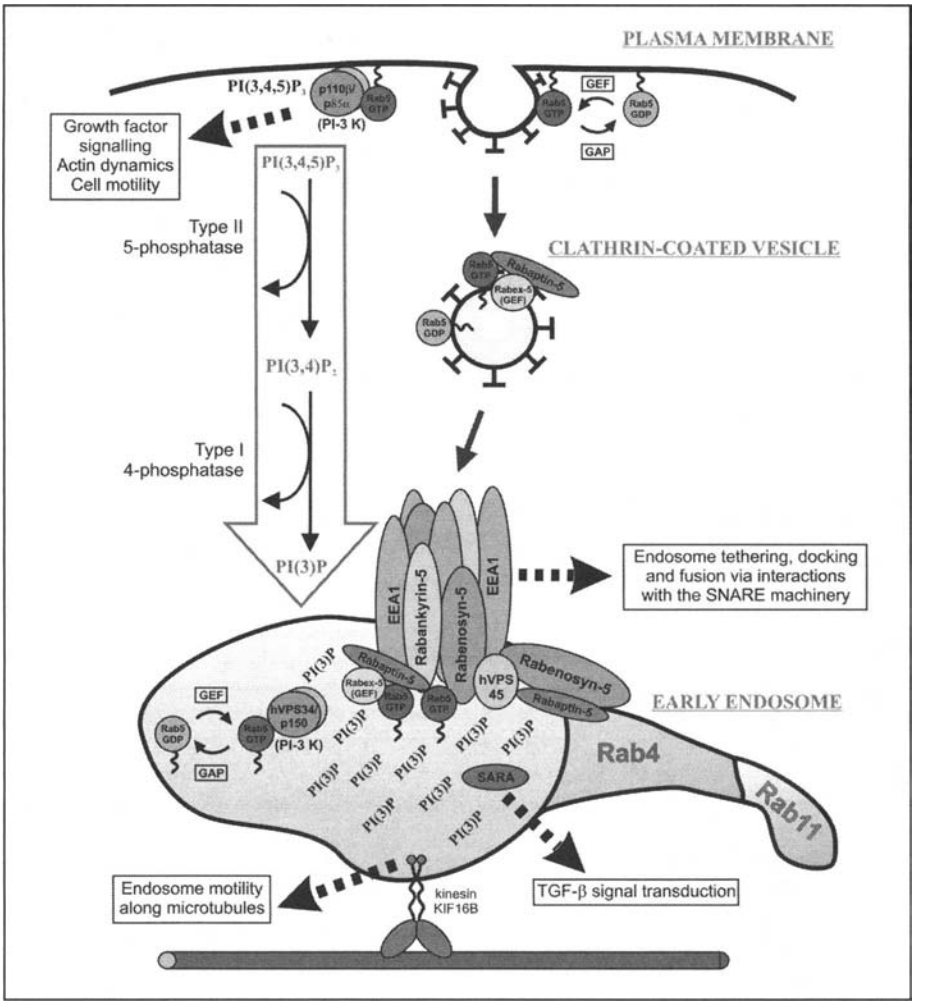

Figure 2. Interactions between Rab5 and its effectors in the endocytic pathway. Rab5 interacts with different effectors on the plasma membrane, clathrin-coated vesicles and early endosomes. The enzymatic cascade of Rab5 effectors which leads from $PI(3,4,5)P_3$ at the plasma membrane to PI(3)P at the early endosomes[64] is depicted by a red arrow. The molecular assembly of Rab5-PI(3)P domain on early endosome and its various functions are presented. See text for the detailed description.

in detail yet. PIKfyve, a protein and lipid kinase regulating the morphology of late endosomes through production of PI(5)P and $PI(3,5)P_2$[72] has been shown to interact with and phosphorylate the Rab9 effector p40.[73] As Rab9 regulates trafficking of cargo such as CI-MPR from late endosomes to TGN, the PIKfyve-p40 interaction has been proposed to function in this transport step, again indicating molecular links between the production of specific phosphoinositides and the Rab machinery. Similarly, phosphatidylinositol 4-kinase β (PI4Kβ) has been shown to interact with the active form of Rab11.[74] However, as Rab11 is present not only within the endosomal system but also in the Golgi complex,[36] this interaction appears to

be important for the biosynthetic transport from the Golgi to the plasma membrane. Similarly, Golgi-resident PI4K Pik1p in yeast appears to be functionally linked to Rab11-related GTPase Ypt31p in regulating protein trafficking through the secretory pathway, although a direct interaction between the two proteins has not been demonstrated.[75]

Functional connections between lipid-metabolizing enzymes and Rab proteins represent a more general phenomenon observed for other subfamilies of small GTPases which use such enzymes as downstream effectors. Examples include PI3K as Ras effector,[76] phospholipase C-β2 as an effector of Rac[77] or phosphatidylinositol 4-phosphate 5-kinases as ARF6 and ARF1 effectors.[78,79] Although the domain distribution of these GTPases on their target membranes has not been systematically analyzed, it is very likely that their ability to affect the lipid composition of the bilayer leads to the formation of specific domains in a manner analogous to Rab proteins.

Functional Coordination between Components of the Rab5 Domain

The function(s) of Rab proteins are determined by the number and type of interacting with them effectors. Accordingly, single Rab protein capable of binding various effectors can mediate several functions. Rab5 shown to interact with over 30 proteins provides an example of a multifunctional GTPase, regulating several aspects of endocytic membrane transport.[80,81] Initially, over a decade ago, Rab5 has been characterized as a factor required for homotypic fusion of early endosomes and heterotypic fusion of early endosomes with plasma membrane-derived clathrin-coated vesicles.[17,82] A more recent identification of Rab5 effectors[80,81] has not only shed light on the molecular mechanisms of these processes but also uncovered new unexpected functions for Rab5, such as regulation of endosome motility or signal transduction (Fig. 2, see also below).

The involvement of Rab5 in endosomal fusion can be primarily attributed to the regulation of docking, a process preceding SNARE-mediated fusion reaction. Rab5 domains on early endosomes enriched in PI(3)P recruit EEA1 which is a crucial factor for endosome tethering and docking.[80] Moreover, EEA1 appears to form oligomeric complexes with the components of the SNARE machinery such as syntaxin 13, NSF or α-SNAP, thus most likely providing coupling between docking and fusion steps.[42] A second link with SNAREs is provided by Rabenosyn-5, another Rab5 effector recruited to the PI(3)P-enriched domain via its FYVE domain. Rabenosyn-5 interacts with a Sec1-like protein Vps45, which binds several endosomal syntaxins.[58] Interestingly, Rab5-PI(3)P domain on early endosomes seems to act as a docking platform for fusion of incoming clathrin-coated vesicles which contain Rab5 but are devoid of VPS34/p150 activity[54,83] (Fig. 2). Thus, Rab5 domain on early endosomes has an additional role in specifying the directionality of membrane transport from the plasma membrane. Moreover, excessive activation of Rab5 can direct caveolar vesicles, normally following an independent trafficking route, to fuse with early endosomes,[61] arguing that Rab5 controls also transport between caveolae/caveosomes and endosomes.

In addition to the regulation of docking and fusion, the presence of PI(3)P in Rab5 domains is crucial for other processes. One of them is motility of early endosomes along microtubules.[84] Recently, KIF16B kinesin motor containing PI(3)P-binding PX motif has been shown to mediate a Rab5-dependent, plus end-directed movement of early endosomes.[62] Deregulation of KIF16B and the resulting repositioning of early endosomes in the cell significantly affected the transport of endocytic cargo towards degradation or recycling. Thus, Rab5- and PI(3)P-dependent motility of endosomes appears to be crucial for proper endocytic trafficking.

Another process requiring the presence of PI(3)P on early endosomes is signal transduction in response to transforming growth factor-β (TGF-β) mediated by a FYVE-domain protein SARA (Smad anchor for receptor activation).[85] The localization of SARA to endosomes, which depends on PI(3)P and can be disrupted by dominant-negative Rab5 mutant,[86] is required for

downstream signaling events, such as Smad2 nuclear translocation.[87] Interestingly, in addition to being one of the key components of TGF-β signaling cascade, SARA appears to have a second function as an endocytic factor, regulating the morphology of endosomes and transport of transferrin.[88] Such dual role of SARA may not be surprising in the light of tight mutual interdependence between endocytosis and signal transduction, many aspects of which are constantly being uncovered (for recent reviews see refs. 89-91).

Rab5 involvement in signal transduction is not limited to the recruitment of signaling proteins to endosomes, as Rab5 itself is a target of regulation by receptor tyrosine kinases (RTKs) at several levels. The enzymatic activity of Rab5 GEF RIN1 or GAP RN-tre can be modulated by growth factors such as epidermal growth factor (EGF), thus subjecting Rab5 nucleotide cycle and the resulting changes in endocytic rates to the regulation by signaling cascades.[48,52,69] Moreover, Rab5 seems to be required for RTK-induced actin remodeling in a process mediated by RN-tre.[92] A recent identification of APPL proteins as Rab5 effectors uncovered yet another role for Rab5 in transduction of signals from the plasma membrane to the nucleus.[71] APPL proteins are signal transducers required for cell proliferation, with a dual localization on endosomal membranes and in the nucleus. Interestingly, APPL-containing Rab5 endosomes appear to be distinct from the PI(3)P-positive compartments and preferentially accessible for certain cargo such as EGF but not transferrin, indicating that Rab5 may be involved in biogenesis of various endocytic structures besides canonical, PI(3)P-containing early endosomes.

Dynamic Coupling between Rab Domains

In order to regulate cargo transport along the endocytic routes, Rab domains need to be functionally linked. Transport of cargo to consecutive compartments, with an ultimate goal of degradation or recycling, appears to be achieved via sequential transfer between neighboring Rab domains (Fig. 1). As described above, transferrin is internalized into Rab5 domains and recycled passing through Rab4 and Rab11 domains.[34] At the molecular level, coupling between Rab domains is provided by two main mechanisms: 1) bivalent effectors, binding active forms of two Rab proteins, and 2) Rab-dependent recruitment of GEFs. The first example of a coupling protein was provided by Rabaptin-5, discovered to interact with both Rab5 and Rab4.[93] Subsequently, other cases of bi-functional effectors have been identified and include Rab4 and Rab5 effectors Rabenosyn-5[94] and Rabip4';[95] Rab5 and Rab22 effector EEA1;[37] Rab4 and Rab11 effector Rab Coupling Protein RCP[96] or Rab5 effector hVPS34/p150[54] potentially interacting also with Rab7.[97] These molecules can regulate the morphology and functionality of Rab domains, arguing that coupling between them is not permanent but can be dynamically modulated. Indeed, overexpression of Rabenosyn-5 has been shown to increase the association between Rab5 and Rab4 endosomal domains, at the same time decreasing the fraction of Rab4- and Rab11-positive structures and resulting in a changed kinetics of transferrin recycling.[94] Although not directly demonstrated, it is plausible that hVPS34/p150, which generates PI(3)P, could link Rab5 and Rab7 domains to ensure transfer of cargo from early to late endosomes towards degradation. Interestingly, the presence of PI(3)P in Rab5 and Rab7 domains could result in recruitment of PIKfyve, which possesses itself a PI(3)P-binding motif and uses this lipid as a substrate for production of $PI(3,5)P_2$, characteristic of late endosomes.[72]

A second mechanism for sequential coupling of Rab domains has been uncovered by another study proposing that Rab proteins within yeast secretory pathway act in a cascade[98] with a preceding Rab recruiting a GEF to activate the consecutive one. In the described case, active Ypt31/32p present on the Golgi membranes bind their effector Sec2p which in turn acts as a GEF for another Rab GTPase, Sec4p.[99] While Ypt31/32p have been implicated in the intra-Golgi transport and budding of secretory vesicles from the Golgi membrane,[100,101] Sec4p regulates fusion of these vesicles with the plasma membrane.[102] By interaction with Ypt31/32p, Sec2p gets incorporated into secretory vesicles and ensures activation of Sec4p, which is required for vesicle exocytosis. An analogous mechanism could potentially act at various steps

of endo- and exocytosis also in higher organisms, although further evidence in support of such model is currently lacking.

Future Prospects

The concept of Rab domains provides a framework explaining the organization of the endocytic organelles. However, several questions are posed by this model. In terms of molecular mechanisms, one of the key problems is the size and the temporal stability of Rab domains. They could represent relatively stable entities, able to dynamically grow or shrink but retaining a minimal steady 'core'. In such case, cargo would be sequentially transferred between the preexisting Rab domains. An alternative model envisages that Rab domains could be periodically disassembled and assembled de novo. In this option, consecutive Rab domains would be sequentially formed during transport of cargo on the membrane encapsulating it. Very recently, both models received some initial experimental support[103,104] but further careful quantitative imaging of endocytic transport in living cells should shed more light on the dynamics of Rab domains in time with respect to cargo flow. A related issue, which needs to be addressed as the visualization methods are improved in the future, is the size range of individual Rab domains and which consequences the domain size may have for the regulation of membrane flow.

Only a limited number of endocytic Rab proteins have been characterized in detail with respect to their exact intracellular localization, domain formation and interacting effectors, although potentially a large number of Rab proteins may regulate endocytosis. It is unclear at present whether all of them are able to actively form specialized membrane domains, like Rab5, or whether some of them only cosegregate within the already existing Rab domains. For example, it will be interesting to see whether Rab22 exhibiting the highest sequence homology to Rab5 and interacting with EEA1 similarly to Rab5[37] can form a separate domain on early endosomes. Extending this question, future studies should reveal whether Rab proteins expressed only in certain cell types are capable of forming specialized membrane domains or even whole specialized organelles in their target cells and whether they could do so also when introduced in a heterologous system. Systematic characterization of other Rab proteins will undoubtedly lead to a more complete picture of all Rab domains present in the cell and their mutual relationships.

Finally, it remains to be determined to what extent small GTPases from other families, such as Ras, Rho or Arf, act as organizers of membrane domains and how such domains could relate to Rab domains. Clearly, signals mediated by various GTPases, such as Ras-mediated signal transduction, Rho-dependent cytoskeleton rearrangements or Arf-regulated budding events, need to be functionally integrated with the membrane flow orchestrated by Rab proteins. Indeed, dual-specificity effectors binding GTPases of different classes have already been identified, such as Arfophilins which regulate the distribution of recycling endosomes and interact with Rab11 and Arf5[105] or the Exocyst complex binding a variety of GTPases of Rab, Rho, Ral and Arf subfamilies through its various components.[106-108] Further studies of small GTPases and their effectors will be pivotal for our understanding of how membrane compartmentalization into domains may specify the identity and function of intracellular organelles.

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CHAPTER 4

Synaptic Endosomes

Oleg Shupliakov*[1] and Volker Haucke[1]

Abstract

Endosomes are important functional elements of the chemical synapse. They are used in membrane trafficking pathways controlling recycling and degradation of pre- and post-synaptic membrane proteins. Recent data indicate that they play a role in maintaining the pool of small synaptic vesicles and are involved in recycling of dense-core vesicle membrane during neurotransmitter release.

Membrane Trafficking Events at Synapses

Membrane trafficking in nerve cells appears to be more complex than in most other cell types. In addition to pathways common for nonneuronal cells, these cells utilize membrane trafficking mechanisms to release neuroactive substances into the surrounding environment.[1,2] These events occur to a large extent in specialized intracellular contacts established by neurons on target cells. These junctions are referred to as chemical synapses.

Chemical synapses are specialized signaling units composed of a pre- and a post-synaptic element. The postsynaptic element contains neurotransmitter receptors and protein machineries involved in signaling and receptor trafficking (see below). The presynaptic nerve terminal, in addition, contains neurotransmitter-filled organelles (vesicles), which may fuse with the presynaptic membrane. Neurons can secrete a variety of nonpeptidergic/classical and peptidergic transmitters via at least two types of secretory organelles, the small synaptic vesicles (SSVs) and the dense-core vesicles (DCVs), also referred to as secretory granules (Figs. 1 and 2A, B). According to the current model, the classical neurotransmitters acetylcholine (ACh), noradrenaline (NA), glutamate, glycine, and GABA are released from SSVs.[2] Neuropeptides, on the other hand, are stored in, and released from, DCVs,[3] which are directly formed at the trans-Golgi network and transported down the axon to their release sites.

Exocytosis of SSVs and DCVs is differentially regulated and takes place at different release sites of the nerve terminal.[4,5] SSVs empty their content upon depolarization and fusion of synaptic vesicles at defined regions of the presynaptic membrane. These areas contain a high density of calcium channels and protein complexes involved in vesicle docking and fusion and are referred to as "active zones".[2] DCVs tend to fuse outside the active zone region. Following neuroexocytosis, neurotransmitter molecules bind to postsynaptic receptors leading to an electrical response of the postsynaptic neuron. In addition, neurotransmitter release may lead to an activation of presynaptic receptors, which control retrograde modulation of neurotransmitter release. Recent data clearly demonstrate that receptors located on pre- and

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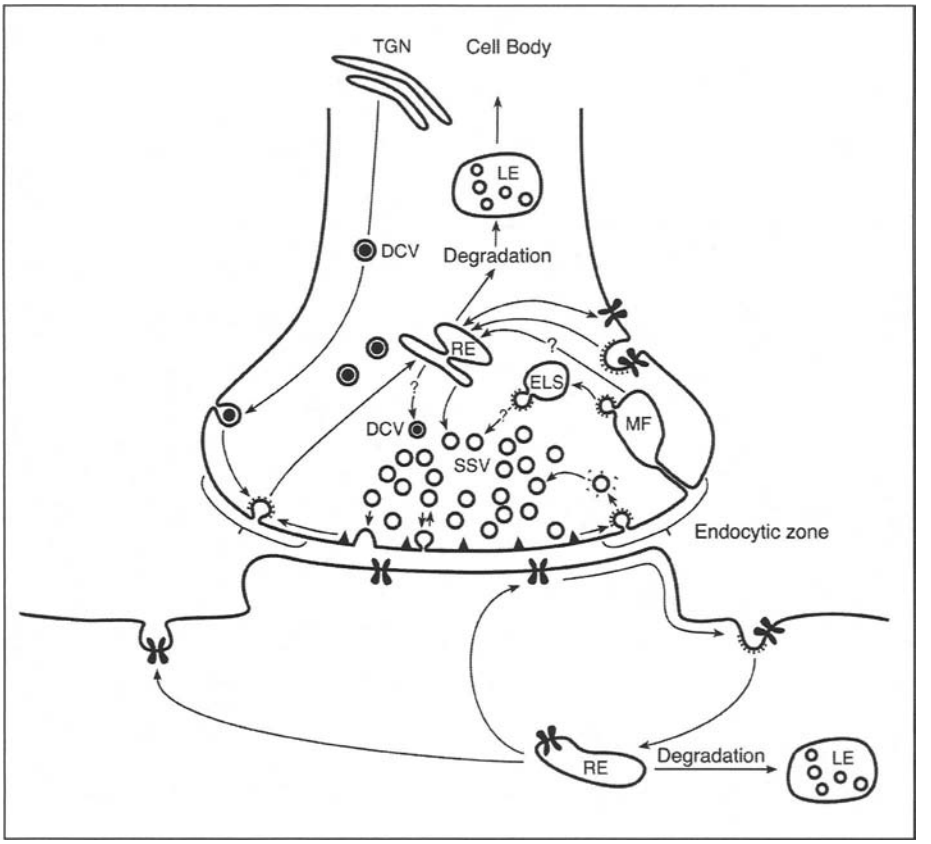

Figure 1. Membrane-trafficking pathways in synapses. Small synaptic vesicles (SSV) releasing neurotransmitter at the active zone (triangles) may be retrieved via "kiss and run" as well as via a clathrin-mediated mechanism. During intense activity, deep plasma membrane folds (MF) and endosome-like structures (ELS) are generated. Dense-cored vesicles (DCV) are synthesized at the TGN and are transported from the cell body to the synapse. They fuse predominantly outside the active zone. Their membrane may be retrieved by clathrin-dependent mechanisms. The 'classical' recycling pathway at synapses may regulate internalization and surface expression of receptors, transporters, and ion channels within both pre and post-synaptic compartments. This pathway involves early recycling endosomes (RE) as well as late endosomal compartments (LE, also referred as to multivesicular bodies), if a receptor undergoes degradation. Links in the pathways marked (?) remain to be elucidated. Depending on the physiological conditions, postsynaptic receptors may be retained in REs or targeted to late endosomes (LE) for degradation resulting in activity-dependent long-term depression (LTD). Under conditions favoring long-term potentiation (LTP) receptors can be exocytosed in an activity-dependent manner from recycling endosomal pools (RE) to extrasynaptic sites at the cell surface from where they are shuttled laterally to the synapse.

post-synaptic membranes can be retrieved from, or exposed at the membrane surface via distinct membrane-trafficking mechanisms, which may underlie synaptic plasticity phenomena.[6]

Several membrane retrieval mechanisms may function in a synaptic terminal. These involve uptake of membrane components related to SSVs, DCVs (see below), as well as receptors and ion channels. The latter mechanism resembles internalization of nutrient and signaling receptors in nonneuronal cells described in other chapters of this book.

Endocytic Recycling of Presynaptic Vesicles

Three mechanisms for synaptic vesicle endocytosis have been proposed: direct reformation of vesicles via the rapid closure of a transient fusion pore ("kiss-and-run"), clathrin-mediated endocytosis,[7] and bulk endocytosis.[8,9] During "kiss-and-run", SSVs are hypothesized to make brief contact with the plasma membrane forming a transient fusion pore through which the neurotransmitter is released.[2,10,11] In contrast, clathrin-mediated endocytosis occurs after complete fusion of SSVs with the plasma membrane.[2,12,13] The key components of the clathrin-dependent endocytosis machinery are: clathrin, the heterotetrameric adaptor complex (AP-2), and dynamin.[2,14] These proteins are part of the coat complex from very early stages. Recruitment of AP-2 to the plasma membrane is a complex process, which involves interactions with phosphoinositides, synaptotagmin,[10,15,16] and accessory proteins.[16] Although synapses use basically the same clathrin-dependent endocytic mechanism as nonneuronal cells, they utilize protein isoforms, most highly expressed in neurons. These include for example: AP180, auxillin, intersectins, dynamin-I, adaptin and the splice-variants of clathrin light chains.[2,14,17,18] AP180/CALM, epsins, intersectin and HIP1/HIP1R (huntingtin interacting proteins) function as cargo adaptors in addition to AP-2 (see chapter 10). While the clathrin lattice is formed, endophilin, epsin, and amphiphysin are involved in membrane invagination and clathrin rearrangements.[2,14,18] The GTPase dynamin is required for fisson of endocytic membrane vesicles.[17] Observation of clathrin-coated pit dynamics using total internal reflection microscopy indicates that during fission, dynamin recruitment to coated pits is rapidly followed by recruitment of actin.[19] Moreover perturbation of actin disrupts the endocytic reaction with accumulation of coated pits with wide necks[20] suggesting a role of actin and dynamin-interacting accessory proteins in promoting constriction of the neck. In lamprey, snake, and fly neuromuscular synapses, the invagination of the membrane into pits occurs at distinct "endocytic zones" surrounding the active zones of exocytosis. Distinct "hot-spots of endocytosis" have been also described at the post-synaptic membrane (Figs. 1 and 5; see also refs. 12,21).

Deep plasma membrane expansions and endosome-like compartments have been observed in synaptic terminals close to active zones during high-frequency stimulation of neuromuscular junctions, retina and also in central synapses.[7-9,22] They could be clearly seen in the lamprey giant synapse. The active zone in this junction is surrounded by organelle-free axoplasmic matrix. This allows following of these structures in serial ultrathin sections using electron microscopy (Fig. 2 C-I). Nonspecific membrane internalization by bulk endocytosis may prevent expansion of the cell surface under conditions in which clathrin-mediated endocytosis becomes rate limiting. Clathrin-coated buds are present not only at the plasma membrane but also on such endosome-like invaginations in synapses, consistent with the existence of the parallel pathway for clathrin-dependent synaptic vesicle formation (Figs. 1 and 2).

Presynaptic Endosomal Compartments

In analogy to nonneuronal cells endocytic vesicles presumably fuse with an endosomal compartment after detachment from the plasma membrane. Several studies performed recently support the involvement of bona fide endosomes in synaptic membrane recycling, although their role in different pathways still remains a matter of debate.[2,23] In hippocampal synapses for example, the role of endosomes in SSV recycling until recently had been believed to be limited. Studies using the fluorescent membrane dye FM1-43 have demonstrated that the amount of dye per vesicle taken up by endocytosis equals the amount of dye a vesicle releases upon exocytosis. It was thus concluded that the internalized vesicles participating in endo-exo recycling do not communicate with intermediate endosomal compartments during the recycling process.[24] These experiments, however, did not exclude the possibility that a remaining, "second" population of vesicles, not participating in exo-endocytic recycling, could exchange membrane with an endosome or that this organelle could be recruited upon certain activity demands. Several recent studies have provided evidence that the population of SSVs is

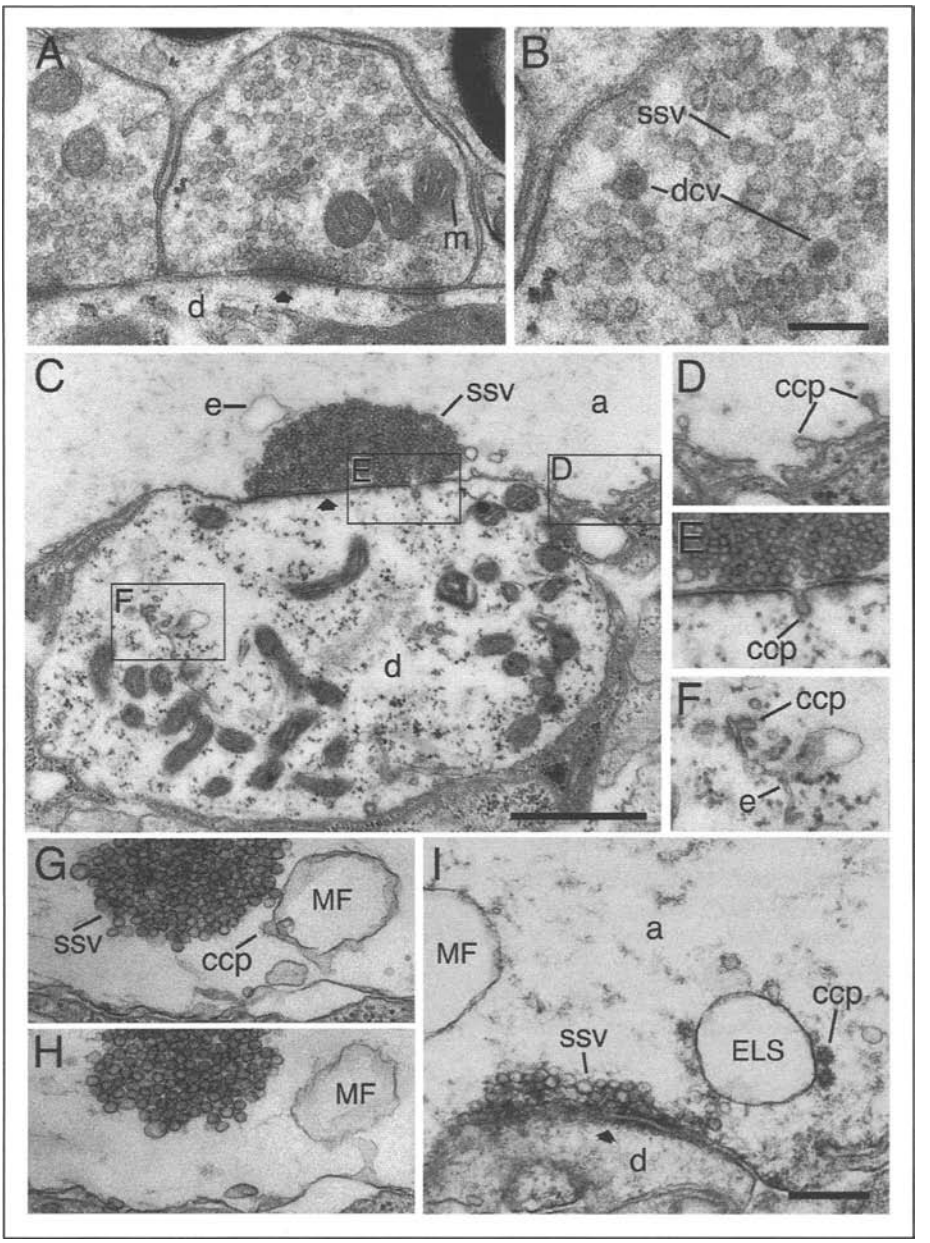

Figure 2. Ultrastructure of endocytic intermediates. A) Electron micrograph of a chemical synapse from the cat spinal cord. An area of the terminal containing small synaptic vesicles (SSV) and dense-core vesicles (DCV) is shown in (B) at higher magnification. C) A reticulospinal synapse in the lamprey spinal cord fixed during recovery after high-frequency stimulation. Boxed areas show clathrin-mediated intermediates budding from the presynaptic endocytic zone (D), from the post-synaptic membrane (E), and from an endocytic compartment of the post-synaptic dendrite, d (F)je-putative endosome. G and H) Serial ultrathin sections from the endocytic zone of a lamprey giant synapse stimulated with high K^+ (30 mM) showing a plasma membrane fold

(MF) containing a clathrin-coated intermediate (ccp). (I) Electron micrograph of a giant synapse stimulated with high K^+. Note the endosome-like compartment (ELS; as revealed from serial sections), containing elathrin-coated intermediates on its surface. Designations: a-axoplasmic matrix; m- mitochondrion. Thick arrows indicate active zones. Bars: A, D-I - 300 nm; B - 200 nm; C - 500 nm. (Shupliakov, unpublished observations; see also Gad et al., 1998).

indeed inhomogenous, consistent with the idea that different pools of vesicles may use distinct membrane-trafficking pathways during the synaptic vesicle cycle. It has been shown, that during development, vesicular release along growing axons of frog motoneurons in culture is sensitive to brefeldin A (BFA), whereas quantal release from nerve terminals is BFA-insensitive.[25] It cannot be excluded that a similar mechanism may be retained in adult synapses. Studies in hippocampal synapses, for example, show that spontaneously recycling vesicles and activity-dependently recycling vesicles originate from distinct pools with limited crosstalk with each other.[26]

Early Endosomal Compartments

Recent studies at the *Drosophila* neuromuscular junction have provided direct support for the involvement of an endosomal pathway in the synaptic vesicle cycle.[27] For a number of years it has been known that the small GTPase Rab5 is present on isolated SSVs.[23,27-29] By recruitment of several effector molecules Rab5 promotes the formation of endosomes in nonneuronal cells.[30,31] Active Rab5 recruits two phosphatidylinositol-3-kinases, PI(3)-kinases p85/p110 and VPS34/p150, which trigger a local enrichment of phosphatidylinositol- 3-phosphate, PI(3)P, in the endosomal membrane.[32] PI(3)P specifically binds to the FYVE zinc-finger domain of endosomal factors such as the Rab5 effectors EEA1 and Rabenosyn-5, which ultimately mediate endocytic vesicle tethering and fusion with early endosomes.[33-36] Consistently, blocking of PI(3)-kinases with antibodies or wortmannin impairs the association of FYVE domain proteins with early endosomes thereby, blocking endosomal membrane trafficking.[35,37] FYVE domains binds to PI(3)P within an intact lipid bilayer[38,39] and the localization of a myc-tagged tandem repeat of the FYVE domain (myc-2xFYVE) is restricted to early endosomes and the internal membrane of multivesicular bodies.[40] Thus, both Rab5 and 2xFYVE can be considered as selective markers for PI(3)P-containing endosomes. Using these two GFP-tagged markers as well as antibodies, González-Gaitán and colleagues have recently demonstrated the presence of Rab5-positive, PI(3)P-containing endosomes at the presynaptic terminal of *Drosophila* neuromuscular junctions.[27] Under conditions in which the SSV pool was depleted, endosomes were drastically reduced in size and recovered by dynamin-mediated endocytosis. Interfering with Rab5 function using a dominant-negative version of Rab5 caused a reduction in the number of released quanta during synaptic transmission, whereas elevated levels of Rab5 increased the quantal content. These data indicate that Rab5-dependent trafficking pathway plays a role in presynaptic vesicle cycling.

Support for the involvement of an early endosomal compartment in synaptic membrane trafficking also comes from studies on the endosomal membrane adaptor complex AP-3. AP-3, which exists as both ubiquitously expressed AP-3A as well as a neuron-specific AP-3B isoform, is localized to the TGN and/or endosomal compartments. It participates in trafficking to the vacuole/lysosome in yeast,[41,42] flies,[43-45] and mammals.[46,47] AP-3B as well as ADP ribosylation factor 1 (ARF1)[48,49] are required for the biogenesis of synaptic-like microvesicles budding from PC12 cell endosomes.[49-52] Genetic analysis of AP-3 mutant mice has been linked to a variety of neurological defects.[50,53-55] The *mocha* mouse, a null mutation of the δ subunit of AP-3, exhibits balance and hearing problems, is hyperactive, and is prone to seizures.[50,53-55] Mice in which the neuron-specific AP-3B subunit μ3B has been genetically deleted, show specific defects related to the biogenesis of GABA-containing SSVs suggesting a particularly important function for AP-3B at inhibitory synapses.[56]

Another potential function for endosome-derived synaptic vesicles and AP-3 dependent pathway is in the recovery of membrane components of dense core vesicles (DCVs) that have

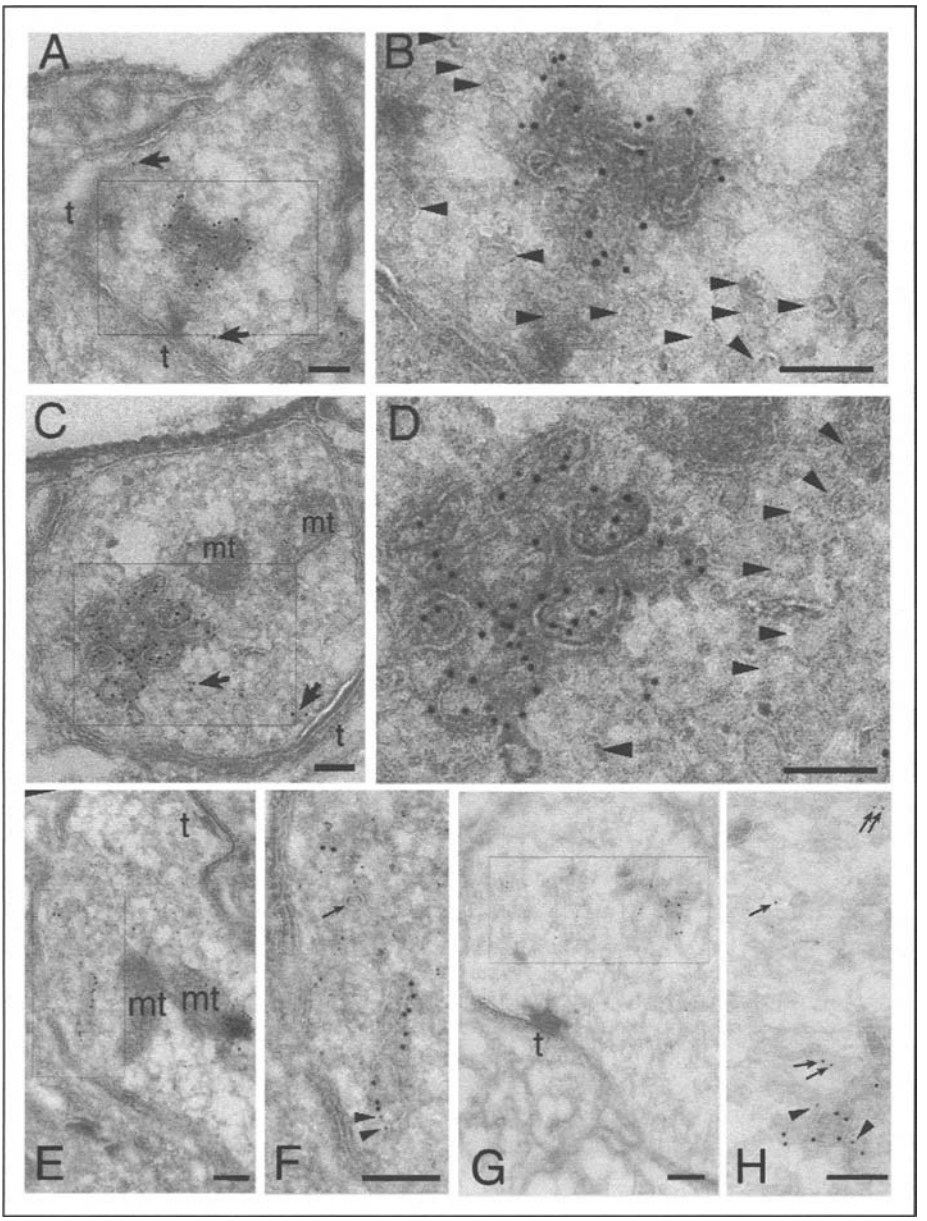

Figure 4. Cryoimmuno-EM of the GFP-2xFYVE endosome. A-D) Cryoimmuno-electron micrographs showing two *Drosophila* presynaptic terminals (A/B and C/D), where GFP-2xFYVE is labeled by 10-nm gold particles (anti-GFP antibody). B and D) High magnifications of the boxes in A and C, respectively. We found cisternal structures of around 150 nm associated to a more electron-dense region within the terminal. The darker regions allow a better contrast for visualization of the membrane (which appear lighter in cryosections) associated to the endosomes, compared with the vesicles with a diameter of 35 or 70 nm. Vesicles are, however, occasionally observed (arrowheads). Only few gold particles (7.8±1.3%, n= 5 sections) are associated with the vesicles (arrows). Cryoimmuno-electron micrographs showing localization of GFP-2xFYVE

(10-nm gold particles) and endogenous CSP (5 nm gold; E and F), or endogenous Rab5 (5 nm gold; G and H). F and H) High magnifications of the boxes in E and G, respectively. E and F) CSP appears throughout the bouton area associated with the pool of vesicles, whereas GFP-2xFYVE is largely restricted to the cisternal endosomal compartments. Although not many vesicles are distinguishable (F, arrow), their presence is revealed by staining of SV integral membrane protein CSP. Few 5-nm gold particles labeling CSP could also be observed in the cisternal structures (F, arrowheads). Rab5 appears in the cisternal structures, (H, arrowheads) as well as in other regions corresponding to vesicles or cytosol (H, arrows). t, T-bar or electron-dense regions indicating active zones; mt, mitochondria. Bars: (A-D) 150 nm; (E-H) 200 nm *(reproduced with permission from Wucherpfennig et al., 2004).*

just undergone exocytosis. Membrane retrieval of this type has been followed in PC12 cells transfected with a chimeric P-selectin.[52,57,58] It had been proposed that, neuronal AP-3B may recapture protein components of DCV proteins. A recapture step could sequester selected DCV proteins from a degradative pathway and allow them to be incorporated into the synaptic vesicle cycle. The distribution of neuronal AP-3B showed some resemblance to that reported for chromogranin A, a marker of dense core granules, particularly in the stratum oriens and the molecular layer of the dentate gyrus.[59] These data indirectly support a role for AP-3B in the recovery of DCV-derived membrane components.

Late Endosomal Compartments

A more acidic late endosomal compartment has been shown to form during maturation of early endosomes in nonneuronal cells.[1] Whereas early endosomes tend to be tubular and are located towards the cell periphery, late endosomes are more spherical and often appear closer to the nucleus. A subset of late endosomes has a multivesicular appearance, hence named multivesicular bodies (MVBs). Late endosomes form a dynamic network together with lysosomal structures, the end point of endocytosis and site of protein degradation. As mentioned above, transport of DCV membrane constituents in neurons involves early endosomes,[58] whereas multivesicular bodies may particpate in retrograde transport of DCV components towards the cell body. Such transport has been observed i.e., in the splenic nerve.[60]

Endosomes in Postsynaptic Receptor Trafficking

Over the past few years it has become clear that the strength of synaptic connections, in particular with respect to postsynaptic responses, is subject to plastic changes. At excitatory synapses, activation of glutamate receptors, such as AMPA-type glutamate-gated ion channels provides the primary depolarization in excitatory neurotransmission. AMPA receptor-mediated postsynaptic currents are modulated by changes in their localization and surface expression. Glutamate receptor density thus appears to be carefully regulated by fine-tuning receptor synthesis, endosomal trafficking, and degradation.[6] Since most of what we know about endosomal trafficking of postsynaptic receptors has been derived from studies on excitatory glutamate receptors we will focus primarily on these, but it is expected that similar mechanisms are utilized for other receptor types as well. In agreement with this notion it has been reported that ionotropic $GABA_A$[61-63] and glycine[64,65] receptors regulating inhibitory neurotransmission in the nervous system, can also be internalized into endosomal or subsynaptic compartments.

AMPA Receptors Are Internalized via Constitutive or Ligand-Induced Pathways

AMPA receptors, heterotetramers composed of related GluR1-4 subunits, undergo dynamic redistribution in and out of the postsynaptic membrane. Most excitatory synapses form on dendritic spines, that emanate from the main shaft and usually bear a single synaptic contact at their heads. AMPA receptors, concentrated at the postsynaptic density (PSD) of dendritic spines, serve to propagate the signal[66] and are able to dynamically move into and out of the postsynaptic density by lateral diffusion. They may also undergo constitutive internalization.[67]

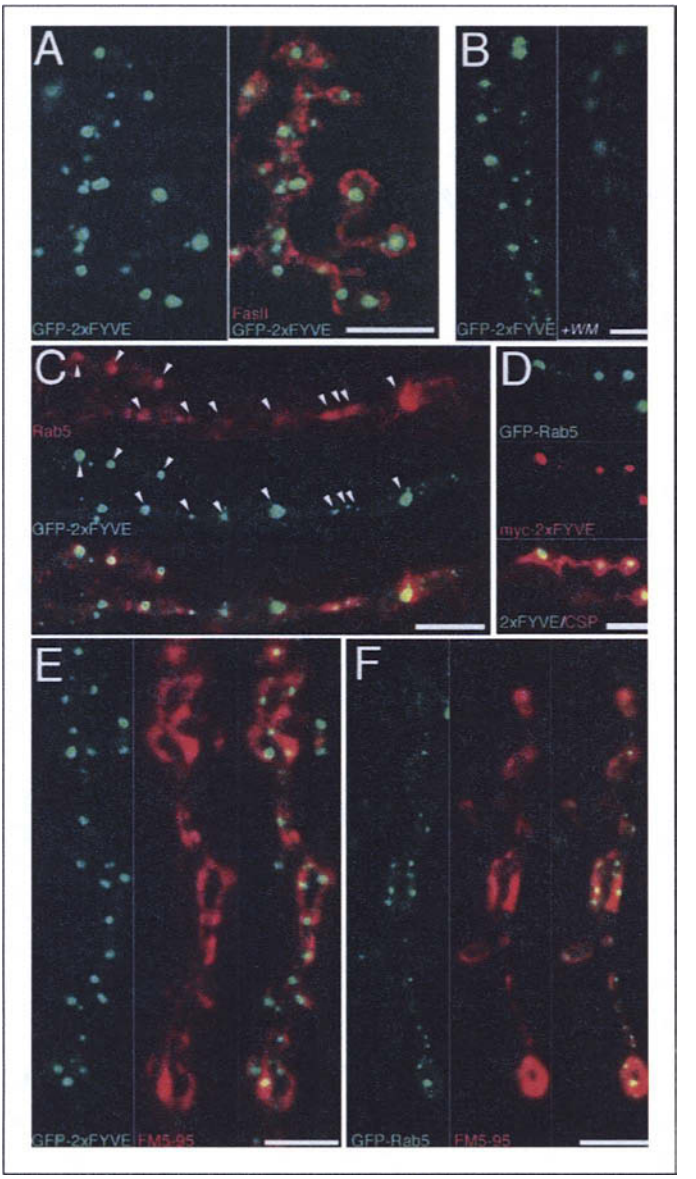

Figure 3. An endosomal compartment at the presynaptic terminal. A) Double labeling showing GFP-2xFYVE (green) to monitor the endosomes and Fasciclin II immunostaining to label the NMJ presynaptic terminals (FasII, red). B) GFP-2xFYVE fluorescence in an abdominal muscles 6/7 NMJ before (left) and after (right) a 45min treatment with 100 nM wortmannin in vivo. Note that, upon wortmannin treatment, GFP-2xFYVE loses the punctate pattern and becomes dispersed into the cytosol. Untreated controls retained the punctate pattern. C) Double labeling showing endogenous Rab5 immunostaining (red) and GFP-2xFYVE (green); lower panel shows merge. Arrowheads indicate Rab5 punctate structures colocalizing with GFP-2xFYVE-positive endosomes. Notice also that some of the Rab5 endosomes do not contain GFP-2xFYVE, consistent with two

types of Rab5 endosomes, EEA1 positive/negative. D) Triple labeling showing GFP-Rab5 (top, green), endosomal myc-2xFYVE immunostaining using an anti-c-myc antibody (middle, red; bottom, green) and CSP immunostaining (CSP, bottom, red) to label the presynaptic terminals in a muscles 6/7 NMJ. Bottom panel is a merge of myc-2xFYVE and CSP. GFP-Rab5 and myc-2xFYVE show a complete colocalization. Double labelings in green (E) GFP-2xFYVE or (F) GFP-Rab5 and in red FM5-95 styryl dye internalized into the presynaptic terminal upon a 1-min stimulation with 60 mM K^+ to label the pool of recycling vesicles (E and F) in two different abdominal muscles 6/7 NMJs. Right panels show merge. Note that the endosomes are embedded within the pool of recycling vesicles. NMJs from late third instar larvae. Genotypes: (A-C and E) *w; UAS-GFP-myc-2xFYVE; elav-GAL4;* D) *w; UASmyc- 2xFYVE/elav-GAL4 UAS-GFP-Rab5;* and (F) *w; elav-GAL4/UASGFP-Rab5.* Bars, 5 μm. *(reproduced with permission from Wucherpfennig et al., 2004).*

Stimulation of glutamatergic synapses with AMPA, NMDA, or insulin has been shown to enhance AMPA receptor internalization by clathrin-mediated endocytosis.[67-71] AMPA receptor internalization along the endocytic pathway correlates physiologically with activity-dependent long-term depression (LTD). Conversely, during long-term potentiation (LTP), a cellular model for learning and memory, an increase in the number of functional, cell-surface exposed AMPA receptors at the postsynaptic membrane is observed (Fig. 1; see also refs. 6,72). These receptors are thought to originate from an intracellular reserve pool.[73,74] Endocytic removal of AMPA receptors occurs mostly from extrasynaptic sites.[75] This observation is consistent with the predominant localization of endocytic proteins including clathrin, AP-2, and dynamin lateral to the postsynaptic density.[76]

The exact molecular mechanisms of the constitutive and regulated pathways for AMPA receptor internalization are not yet completely understood. Although all pathways are dependent on the GTPase dynamin, an accessory protein required for fission of both clathrin- and nonclathrin-coated vesicles, and its SH3 domain-containing binding partners,[67] they seem to be spatially segregated and differentially influenced by protein kinases,[77] phosphatases, and calcium ions.[68,70]

AMPA Receptors Undergo Differential Endosomal Sorting

Different stimuli differentially affect the subcellular localization of internalized receptors. AMPA receptors endocytosed via direct agonist stimulation (i.e., AMPA) colocalize with early endosomal markers such as early endosomal antigen 1 (EEA1), syntaxin 13, and endocytosed transferrin receptors. In contrast, AMPA receptors internalized via insulin- or NMDA-regulated signaling pathways although initially present in EEA1-positive early endosomes appear to segregate into distinct compartments, which may include late endosomes and lysosomes;[78] but see[77] for a different view). How precisely and at which stage differential endosomal sorting occurs remains unclear. Activated AMPA receptors colocalize with AP-2[69] and Eps15[67] in clathrin-coated pits. Direct binding of the basic stretch within the cytoplasmic tail of the AMPA receptor, subunits GluR1-3, to the clathrin adaptor complex AP-2 is only required, for NMDA-induced AMPA receptor endocytosis,[79] thus indicating that differential recognition modes at the cell surface may contribute to endosomal sorting. In nematodes, GluR is subject to multi-ubiquitination, which may target glutamate receptors for internalization and late endosomal/ lysosomal degradation.[80] Differential sorting of receptors recognized directly by endocytic adaptors or modified by ubiquitination is seen in nonneuronal cells, i.e., in the case of internalized transferrin vs. epidermal growth factor receptors.[81,82] Additionally, insulin-stimulated AMPA receptor internalization may be regulated by tyrosine phosphorylation,[83] similar to what is seen for growth factor receptors.[81]

Receptor Determinants for Endosomal Sorting

As discussed above AMPA receptors internalized in response to direct agonist binding (i.e., AMPA) or NMDA-induced signaling cascades initially share the same early endosomal sorting

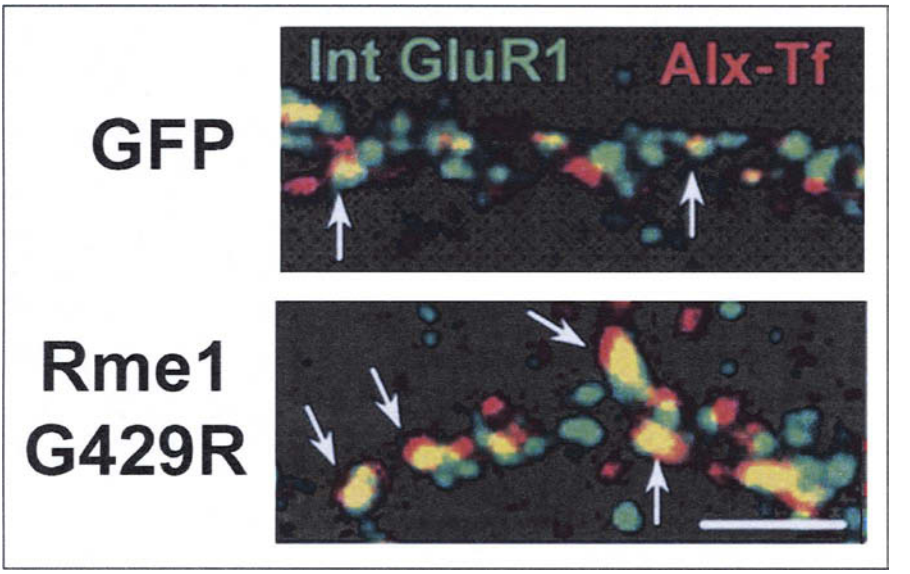

Figure 5. Recycling endosomes supply AMPA receptors for long-term potentiation (LTP) Overexpressing a mutant version of the Eps15-homology domain protein EHD1/ Rme1 (Rme1-G429R) traps internalized AMPA receptor GluR1 (green) in recycling endosomes where it colocalizes (yellow puncta, see arrows) with endocytosed Alexa-labeled transferrin (red). Scale bar, 2 μm. Under such conditions LTP is abolished. *(reproduced with permission from Park et al., 2004).*

pathway.[78,84] During AMPA-induced internalization, homomeric GluR2 receptors appear to be retained within early recycling endosomes, whereas GluR2 endocytosed in response to NMDA is diverted to late endosomes and lysosomes for degradation.[78] One important factor regulating sorting appears to be the subunit composition of heteromeric AMPA receptors. Homomeric GluR1 receptors are retained in recycling endosomes, whereas GluR3 homomers enter the late endosomal/ lysosomal pathway regardless of stimulation.[78] In the context of heteromers endosomal sorting is apparently governed by GluR2, which exerts dominant effects, perhaps by recruiting adaptor proteins,[85] by undergoing posttranslational modifications including tyrosine phosphorylation[83] and ubiquitination[80] or by binding to ubiquitinated adaptor proteins such as PSD-95.[86] In nonneuronal cells, sorting of ubiquitinated cargo is achieved by ubiquitin-interacting motif (UIM) or ubiquitin-associated domain (UBA) containing accessory proteins including the phosphoinositide binding protein epsin, the EH-domain containing endocytic accessory protein Eps15, and Hrs.[81,82] Both epsin and Eps15 are highly expressed in the brain and could serve functions in postsynaptic receptor sorting within the endosomal system, similar to their known roles in presynaptic vesicle recycling. In the case of the inhibitory $GABA_A$ receptor channel, it has recently been demonstrated that huntingtin-associated protein 1 (HAP1) modulates cell surface receptor number by inhibiting lysosomal $GABA_A$ receptor degradation.[87] Since HAP1 can associate with the ubiquitin-binding adaptor Hrs[85] it is tempting to speculate that HAP1 may act by suppressing Hrs-dependent lysosomal receptor targeting. Although HAP1 action appears to be restricted to inhibitory synapses similar regulatory principles may hold true for early endosomal trafficking of glutamate receptors.

Whereas endosomal targeting of AMPA receptors during conditions of long-term depression (LTD) is well established, much less is known about the recycling of internalized receptors to the cell surface. Recent data suggest that indeed recycling endosomes rather than trans-Golgi

network (TGN)-derived vesicles supply AMPA receptors for long-term potentiation (LTP). Blocking exit from recycling endosomes by expression of dominant-negative mutants of either Rab11a or the EH-domain containing accessory protein EHD1/ Rme1 trapped internalized AMPA receptors in recycling endosomes (Fig. 5) and prevented expression of LTP in hippocampal slices.[84]

Thus, early recycling endosomes appear to play crucial roles in synaptic plasticity by regulating the internalization, recycling, degradation, and thus cell surface number of glutamate and possibly other ionotropic receptors at synapses.

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CHAPTER 5

Endosome Fusion

Dorothea Brandhorst and Reinhard Jahn*

Abstract

In recent years it has become apparent that membrane fusion reactions in the secretory pathway are mediated by supramolecular assemblies that include both members of conserved protein families and proteins specific for individual fusion steps. Before fusion, membranes need to recognize and bind to each other, and it is thought that this step is mediated by Rab-GTPases and their effectors. Fusion itself is probably mediated by SNARE proteins but other factors such as SM-proteins are also involved. In this chapter, we discuss fusion reactions of the endocytotic pathway, with particular emphasis on homotypic fusion between mammalian early and late endosomes, and between yeast vacuoles, with a main emphasis on the molecular mechanism of SNARE proteins.

Introduction: Fusion Steps in the Endocytotic Pathway

The endocytic pathway in higher eukaryotic cells comprises pleiotropic intracellular organelles enclosed by single membranes. These organelles are connected with each other by vesicular traffic that includes distinct budding, transport, and fusion steps (Fig. 1, see also Chapter 1). By definition, the starting point of the endocytic pathway is endocytosis, i.e., the formation of invaginations and the pinching off of transport vesicles from the plasma membrane, which include both clathrin-coated and noncoated vesicles, specialized organelles such as phagosomes, and caveolae. With exception of caveolae and phagosomes, the first compartment reached is the early/sorting endosome with which endocytotic vesicles fuse.[1,2] Early endosomes must be considered as an intracellular distribution center from which trafficking pathways lead back to the plasma membrane and to late endosomes/lysosomes. In addition they are connected to the Golgi membrane system. Early endosomes are in dynamic equilibrium and rapidly fuse not only with incoming endocytotic vesicles but also with each other ("homotypic" fusion).[3-5] Steady-state is maintained by the parallel and continuous generation of transport vesicles with different destinations. A first pathway leads directly back to the plasma membrane and mediates recycling of certain receptors. A second pathway involves the formation of cisternal vesicles that are transported to the perinuclear region. They form a separate compartment, termed recycling endosomes, from where membranes and membrane resident proteins are also returned to the plasma membrane, albeit at a slower rate than by the direct pathway. Recycling endosomes also communicate directly with the trans Golgi Network. Third, early endosomes ship vesicles back to the TGN[6] and in turn receive TGN-derived vesicles.[7] Finally, trafficking to late endosomes is thought to occur by maturation of early endosomes that develop into multivesicular bodies/late endosomes.[8,9] Alternatively, it cannot be excluded that early endosomes are more stable compartments that give rise to transport intermediates that are then capable of fusing with late endosomes.[10,11]

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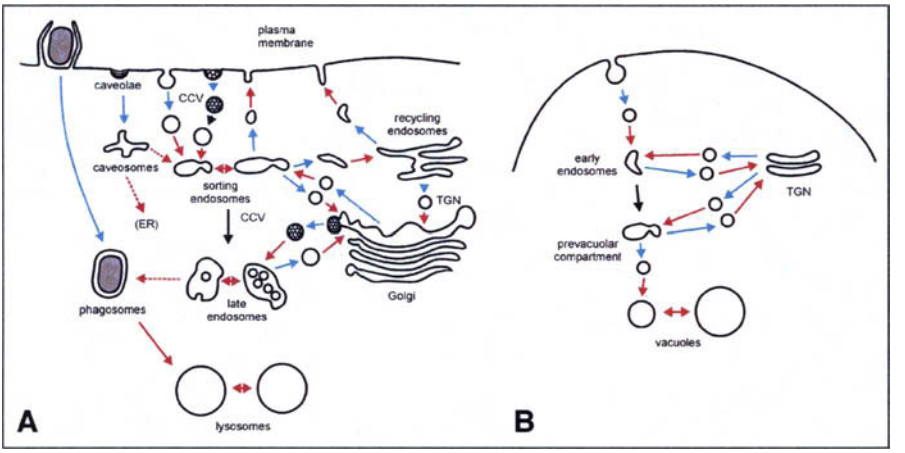

Figure 1. Major trafficking routes of the endocytotic pathway in a mammalian cell (left) and yeast (right). Blue arrows, budding/fission followed by transport; red arrows, transport followed by fusion; black arrows, transport. CCV, clathrin-coated vesicle; TGN, trans-Golgi network. Note that the autophatocytotic pathway is not included.

Like early endosomes, late endosomes are capable of homotypic fusion. Furthermore, they fuse with lysosomes,[12-14] and finally, lysosomes fuse homotypically with each other.[15,16] Late endosomes are also connected by vesicular traffic to the Golgi system.[17,18] It needs to be borne in mind that the list of fusion reactions indicated in Figure 1 may still be incomplete. For instance, in polarized cells apical sorting endosomes do not fuse with basolateral endosomes, suggesting further differentiation of trafficking steps that may also occur in nonpolarized cells.[19] Furthermore, it is becoming apparent that certain cisternal compartments (e.g., early endosomes) are further differentiated into subdomains with distinct functions,[20,21] and it is thus conceivable that a single organelle possesses specialized sites for distinct fusion reactions.

Endocytotic trafficking is also studied intensely in yeast. Although due to the small size of yeast cells imaging approaches are more limited and challenging, the powerful combination of genetics and convenient in vitro fusion reactions led to the discovery of a string of novel proteins, many of which, as it turns out, are conserved from yeast to mammals.[22] Genetics is facilitated by the fact that yeast survives (albeit often poorly) when endocytotic trafficking steps are impaired or completely dysfunctional. In general, the yeast endocytic pathway is similar to that of mammalian cells. It comprises endocytic vesicles, early endosomes, late endosomes that are also termed prevacuolar compartment, and the vacuolar compartment which is considered as equivalent to mammalian lysosomes (Fig. 1B, for review see ref. 23). Fusion reactions take place between endocytic vesicles and early endosomes, between early endosomes and the prevacuolar compartment, and between the prevacuolar compartment and vacuoles. Furthermore, vacuoles fragment during cell division and then coalesce by homotypic fusion. The latter fusion reaction has been intensely studied using both genetic and in vitro approaches.[24-26] Finally, trafficking routes exist between the trans Golgi network and the endosomal membrane system (Fig. 1B).[27,28]

In recent years, we have witnessed an explosive growth in our understanding of intracellular fusion reactions. Conserved protein families have been identified that are responsible for distinct steps in the fusion pathway. While our understanding of some model fusion reactions is already quite advanced, others are much less understood. For instance, it is not known to which extent the fusion reactions indicated in Figure 1 are different at the molecular level. While it is clear that homotypic fusion between early and late endosomes, respectively, involves

different sets of proteins, fusion of endocytotic vesicles with early endosomes appears to share the fusion machinery with homotypic fusion of early endosomes.[29] Another example includes the homotypic fusions of late endosomes and lysosomes, respectively, that appear to involve similar proteins.[30] In these cases, it is not known whether all proteins are shared, and if so, how these fusions are distinguished.

For a successful fusion reaction the membranes need to recognize the appropriate fusion partner, get in close physical contact, and finally fuse their lipid bilayers. The key players in these intracellular fusion reactions are represented by conserved protein families including the small, ras-related Rab/Ypt GTPases, the SM proteins, and the SNAREs. These proteins are interacting with a plethora of additional proteins, many of which are apparently for specific fusion reactions and whose function is often only incompletely understood. In this chapter, we summarize recent developments in the fusion reactions of the endocytotic pathway, with the main emphasis on the fusion reaction itself.

Tethering and Docking

Before fusion, the corresponding organelles need to be brought into close proximity, resulting in physical contact. Little is known about these initial reactions. There are some hints that physical contact between organelles might be sufficient to trigger a rection cascade leading to fusion.[31] Studying tethering and docking requires the availability of in vitro assays, and consequently most of the information is derived from the homotypic fusion of early endosomes in mammalian cells, and from the homotypic fusion of yeast vacuoles. In early endosome fusion, candidate proteins were identified that are likely to be involved in tethering: EEA1 (early endosomal antigen 1) forms long coiled coils that have the capacity to bind to endosomes with two binding sites on both ends and act as long-range tethering factor.[32] EEA1 was found to bind phosphatidylinositol 3 phosphate (PI(3)P) on early endosomes via a FYVE domain and to Rab5. Rab5 belongs to the family of small GTPases that cycle between GTP- and GDP-bound forms. Probably, GTP-Rab5, in conjunction with the local generation of PI(3)P mediates the initial assembly of docking complexes. In addition to EEA1, GTP-Rab5 recruits a plethora of effectors to early endosomes (including a PI-3-kinase) and thereby provides a platform for the assembly of the fusion machinery.[33] This process is discussed in detail in chapter 5 (Zerial).

Homotypic vacuole fusion in yeast in vitro proceeds in four consecutive and discernable steps: priming, tethering, docking and fusion. In the priming step, SNAREs are disassembled (see below, refs. 34,35), a prerequisite for the following tethering reaction. The tethering reaction again involves a Rab-protein (Ypt7p, the yeast orthologue to mammalian Rab7) as central player in the initial steps. Ypt7p is activated by a multiprotein complex referred to as HOPS (homotypic vacuole fusion and protein sorting) or Class C Vps complex that consists of six different proteins termed Vps11p, Vps16p, Vps 18p, Vps 33p (an SM protein, see section *SM-Proteins: Essential but Still Enigmatic*), Vps39p, and Vps 41p.[36-38] Ypt7p activation is needed for docking reactions that interestingly do not seem to involve long tethering proteins such as EEA1. Rather, an involvement of the actin cytoskeleton is suggested by the requirement for activating Rho-GTPases. While tethering by activation of Ypt7 is a reversible process, the subsequent formation of trans SNARE complexes (see below) leads to irreversibly docked vacuoles.[39] Microscopic studies of in vitro docked vacuoles showed that SNARE proteins, the HOPS complex, and Ypt7 are enriched at the rim of the contact sites.[40,41]

The two examples discussed above illustrate the central role of Rab/Ypt proteins in orchestrating docking and tethering. There are about 60 Rabs in mammals and 11 Ypts in yeast.[42,43] Each of them interacts with its own set of partially very diverse effector proteins. While some of these effectors may be shared between subsets of rabs, others are specific. Currently, the Rab/Ypt proteins, together with their effectors, their GTPase activating and guanine nucleotide exchange factors, are the best candidates for determining the specificity of intracellular fusion reactions. Biologically, this makes sense since "proofreading" of the membrane is most efficient

if it occurs early in the reaction cascade instead of late, i.e., when docking machineries are already assembled.

SNARE-Proteins: Central Players in Membrane Fusion

General Properties of SNAREs

SNARE proteins (NSF attachment protein receptors[44] are small (10-35 kDa), mostly membrane bound proteins that each contain a homologous stretch of 60-70 amino acids, referred to as SNARE motif. SNARE proteins undergo an assembly-disassembly cycle. In solution, monomeric SNARE motifs are largely unstructured. When appropriate SNAREs are combined, they form a stable α-helical bundle, called SNARE complex.[45] The crystal structures of the neuronal and the late endosomal SNARE complexes have been solved and show a high degree of structural similarity. They consist of coiled coil bundles that contain four different α-helices.[46,47] The core of the helical bundle is built by 16 layers of highly conserved, mostly hydrophobic amino acid sidechains. Due to a highly conserved polar residue in the center of the SNARE motif which ist either arginine or glutamine, SNAREs are classified into Qa-SNAREs (syntaxins), Qb-SNAREs, Qc-SNAREs and R-SNAREs.[48] Although not conclusively proven in all cases, it is becoming clear that each functional SNARE complex contains one SNARE motif of each of these subfamilies ("QabcR-rule"). The four SNARE motifs can be provided by three different proteins as in the neuronal or by four proteins as in the late endosomal SNARE complex. SNARE complexes show an extraordinary stability towards heat and denaturants, for instance they "melt" only at temperatures above 75°C.[49]

How do SNARE complexes form? The four helices in the SNARE complex are aligned in parallel, with the transmembrane domains at one end and the N-termini at the other end. This led to the so called "zippering model".[50-53] The model proposes that assembly of the SNAREs starts at the N-termini of the SNARE motifs and proceeds towards the C-terminally localized membrane anchor domains. The energy provided by the assembly reaction could be high enough to overcome the repulsion forces of the negatively charged lipid headgroups in the membranes and finally fuse the lipid bilayers. During the fusion reaction, complexes shift from "trans" (transmembrane domains in two different lipid bilayers) to "cis" (transmembrane domains in the same lipid bilayer). According to this model, the function of the SNAREs is to execute membrane merger. Indeed, it has been shown that appropriate sets of SNARE proteins reconstituted into proteoliposomes fuse membranes, albeit at a slow rate.[53-56] However, as discussed further below (see section *Late Steps in the Fusion Pathway and Fusion Catalysis*), this view is not undisputed, and other steps downstream of the SNAREs have been invoked before bilayer fusion.

Disassembly of SNARE complexes is mediated by NSF (N-ethylmaleimide sensitive factor), a hexameric ATPase belonging to the family of AAA proteins (ATPases associated with other activities).[57,58] It requires cofactors called SNAPs (soluble NSF acceptor proteins) for this reaction. In mammals, three isoforms of SNAPs have been identified, α-, β- and γ-SNAP with α-SNAP being the ubiquitous isoform.[59] Three α-SNAPs bind to one SNARE complex, then NSF can bind, resulting in a so called 20S complex.[44] The exact mechanism of SNARE complex disassembly is still unclear. Activation of the NSF ATPase activity by α-SNAP is needed for the disassembly reaction as shown by a dominant negative α-SNAP mutant.[60] A single NSF possesses six identical subunits,[61] each containing a catalytically active ATPase site, thus providing enough energy to disassemble this extremely stable complex. The disassembly of SNARE complexes is essential for fusion, since SNARE proteins are bound in complexes after the fusion reaction and have to be dissociated.

For successful fusion, each membrane has to contain at least one SNARE with a transmembrane domain. In the first characterized SNARE complex involved in neuronal exocytosis, the R-SNARE synaptobrevin is localized to synaptic vesicles while the Q-SNAREs Syntaxin 1 (Qa) and SNAP 25 (Qbc) reside on the plasma membrane. Therefore, SNAREs were

originally classified into v-SNARE (vesicular SNARE) and t-SNAREs (target membrane SNAREs).[44] In many heterotypic fusion events, R-SNAREs are found on vesicles and Q-SNAREs on the target membrane. However, there are exeptions such as the fusion reaction of transport vesicles from the ER with the cis-Golgi in yeast. Here, the vesicle contains R-, Qb- and Qc-SNARE while the Qa-SNARE is found on the target membrane. Furthermore, v- and t-SNAREs cannot be distinguished in homotypic fusion events. In addition, certain SNARE proteins were shown to participate in SNARE complexes with different topologies (see below), further complicating the classification into v- and t-SNAREs (see ref. 62 for a more comprehensive discussion). For these reasons, the structurally based classification into Q- and R-SNAREs is preferable.

SNAREs in the Endosomal Pathway

The original SNARE hypothesis proposed that each individual intracellular fusion reaction is catalysed by a unique set of SNARE proteins that thus would represent the major determinants for the specificity of intracellular fusion reactions.[55,57] However, subsequent work revealed that the situation is more complicated, for the following reasons:

- In vitro studies using purified proteins revealed that SNAREs promiscuously form complexes as long as the QabcR rule is followed although not all of these complexes are as stable as the cognate complexes.[63,64] In liposome fusion experiments, a higher degree of specificity was suggested but these experiments are not conclusive since in many cases the QabcR rule was not followed.[55,65] Promiscuity is in fact not surprising when considering the extraordinary high degree of structural conservation between evolutionary distant SNARE complexes.[46]
- While several lines of evidence indicate that each trafficking step requires specific sets of SNAREs, deletion of individual SNAREs in both yeast and mammals have shown that in some fusion steps certain SNAREs are at least partially redundant. For instance, knock-out mice deficient in the Qb-SNARE vti1b that was previously shown to function in late endosome fusion are viable, and despite pleiotropic phenotypes endocytotic trafficking to the lysosome does not appear to be grossly impaired.[66] Intriguingly, its partner syntaxin 8 (Qc) is downregulated in the absence of vti1b. So either other Qb/Qc-SNAREs are able to substitute, or a second SNARE complex operates in parallel in the same fusion reaction that hitherto has escaped detection. Another example is the R-SNARE Nyv1p that functions in yeast vacuole fusion. Again, deletion results in mild phenotypes,[34] which is probably due to substitution by the R-SNARE Ykt6p.[35]
- Individual SNAREs can participate in several fusion reactions, each involving different SNARE partners. Yeast Vti1p is used throughout the endosomal system as component of four different SNARE complexes, functioning in traffic from the Golgi to the endosome, from the Golgi to the vacuole, in retrotrade traffic to the cis-Golgi, and in homotypic fusion of the TGN.[67] Other examples include the R-SNARE Ykt6p that functions both in homotypic fusion of vacuoles, and in ER-Golgi transport,[35,68] and the R-SNARE Sec22p that functions both in anterograde and retrograde traffic between the endoplasmic reticulum and the cis-Golgi, each step involving different Q-SNARE partners.[69]
- A further complication arises from the fact that as integral membrane proteins, SNAREs need to follow membrane recycling pathways after completing their task in a fusion reaction in order to return to their prefusion compartment. For example, SNAREs involved in exocytosis in neuroendocrine cells recycle through early endosomes. Cleavage of these SNAREs with clostridial neurotoxins has no effect on homotypic fusion[70] suggesting that these SNAREs are not involved. Thus, regulatory factors are needed that distinguish which of the SNAREs to use in an upcoming fusion event and which to silence because they are merely travelling passengers. The nature of these factors is unknown.

Considering these complications it is not surprising that it has been difficult to unequivocally assign SNAREs and SNARE complexes to individual fusion steps of the endosomal

Table 1. SNAREs in endosomal fusion steps

Fusion Step	SNARE Candidates	References
Mammals		
EE-EE/CCV-EE	Qa syntaxin 13, syntaxin16 Qb vti1a Qc syntaxin 6 R VAMP-4/VAMP-8	97,98
LE-LE	Qa syntaxin 7 Qb vti1b Qc syntaxin 8 R VAMP-8	46,72,97
LE-Lys	Qa syntaxin 7 Qb vti1b Qc syntaxin 8 R VAMP-7	14
EE/RE-TGN	Qa syntaxin16, syntaxin 5 Qb vti1a, GS28 Qc syntaxin 6, GS15 R VAMP-4, VAMP-3, Ykt6	99,100
Yeast		
Vac-Vac, Prevac-Vac	Qa Vam3p Qb vti1p Qc Vam7p R Nyv1p, Ykt6p	35,101
TGN-Prevac	Qa Pep12p Qb Vti1p Qc Syn8p R Ykt6p	102

EE: early endosomes; LE: late endosomes; CCV: clathrin-coated vesicles; RE: recycling endosomes; Lys: lysosomes; TGN: trans-Golgi network; Vac: vacuole; Prevac: prevacuolar compartment

pathway, particularly in mammalian cells. Unfortunately, there are no fast-acting tools available that inactivate endosomal SNAREs, unlike exocytotic SNAREs that are selectively cleaved by botulinum and tetanus neurotoxins.[71] Inhibition by antibodies is notoriously unreliable due to steric hindrance, and competition by excess soluble SNAREs is counteracted by the NSF-disassembly reaction, thus further limiting the experimental options for assigning SNAREs to individual reactions.

Table 1 shows the SNAREs that are presently discussed for the fusion steps of the endocytic pathway. It needs to be emphasized that in particular in mammalian cells the evidence for the involvement of a particular SNARE is frequently limited to coprecipitation or to perturbation of in vitro fusion using antibodies and recombinant proteins, and more evidence is required to affirm the involvement of given sets of SNAREs in a particular fusion step.

In mammalian cells, most authors agree that the SNAREs syntaxin 7 (Qa), syntaxin 8 (Qb), syntaxin 8 (Qc), and endobrevin/VAMP8 (R) catalyse the fusion of late endosomes, and a crystal structure for this complex is available.[46,72] However, as discussed above, endosomal trafficking is not blocked in mice lacking vti1b[66] or endobrevin/VAMP8,[73] strongly suggesting the involvement of other SNAREs. For the other fusion steps, the responsible SNARE complexes are even less clear. For instance, for the homotypic fusion of early endosomes the Qa-SNAREs syntaxin 13 and syntaxin16, the Qb-SNAREs vti1a, the Qc-SNARE syntaxin 6,

and the R-SNAREs VAMP-4 and VAMP-8/endobrevin are discussed. Syntaxin 6 and syntaxin 13 were found to bind EEA1.[74,75] These findings suggest a link between SNAREs and the tethering complex orchestrated by Rab5, similar to the link between Ypt7p/HOPS and the SNAREs in vacuolar fusion. On the other hand, syntaxin 16 interacts with the SM protein Vps45 that appears to be specific for early endosome fusion, indicating a role for this Qa-SNARE in the same fusion step.[76] To complicate things further, SNAP-25 has recently been invoked as a Qb/c SNARE in the fusion of early endosomes.[77] SNAP-25 has a well-established role in regulated exocytosis of synaptic vesicles and secretory granules, where it interacts with syntaxin 1 (Qa) and synaptobrevin/VAMP2.[44,57]

In yeast, the evidence invoking specific sets of SNAREs to fusion steps in the endocytotic pathway is stronger. For instance, in homotypic fusion of vacuoles the SNAREs in charge of this reaction include Vam3p (Qa), Vam7p (Q), vti1 (Q), and Nyv1p (R). In addition, Ykt6p can substitute for Nyv1p. Similarly, the identity of the SNAREs involved in fusions at the prevacuolar compartments are better established than those in the correspoinding fusion steps of mammalian cells (Table 1).

SM-Proteins: Essential but Still Enigmatic

SM proteins (Sec1/munc18 like proteins) are hydrophilic proteins of 60-70 kDa that bind to SNARE proteins. Their arch-shaped overall structure seems to be conserved (for review see refs. 78,79). As there are fewer SM proteins than fusion reactions in the cell (7 in the human genome, 4 in yeast), it is assumed that a given SM protein acts in several fusion steps. Fusion is absolutely dependent on the corresponding SM proteins, but the details of their molecular role are not well understood. While most authors agree that SM proteins somehow regulate SNAREs, the mechanism of action is controversial. Furthermore, several SM proteins are—directly or indirectly—linked to Rab/Ypt proteins by means of forming complexes with Rab effectors.

The crystal structure of two only distantly related SM proteins (mammalian Munc18, and yeast Sly1p) show strong structural conservation.[80,81] Furthermore, most SM proteins bind to Qa-SNAREs but surprisingly the nature of the binding interface is not conserved: Syntaxin 1 binds to a large cleft in the SM-protein Munc-18, with syntaxin being folded up in a "closed" conformation.[80] In stark contrast, the Qa-SNARE Sed5p interacts only with few N-terminal amino acids with its SM-partner Sly1p, and binding does not occur to the cleft (that is conserved) but to the outer surface of the globular molecule.[81] In line with these differences, the effects of Qa-SNARESM interaction on SNARE pairing are divergent: Munc18 binding to syntaxin 1 prevents SNARE complex formation, Sly1p or Vps45 binding to the corresponding Qa-SNAREs appears to promote SNARE pairing, and in yet another case (Sec1) the SM protein binds only to the assembled SNARE complex.[78,79,82,83]

Two SM proteins have been identified that operate in fusion events of the endocytotic pathway both in yeast and mammals, including Vps45 and Vps33, and they nicely exemplify the problems in arriving at a common concept for these molecules. Vps45 binds to an N-terminal peptide of syntaxin 16/Tlg2p.[76,84] Syntaxin 16 is one of the Qa-SNARE candidates for an early endosomal SNARE complex (see above). In absence of Vps45p, Tlg2p is no longer capable of forming complexes with its SNARE partners.[85] The Rab5 effector protein Rabenosyn-5 was found to build a complex with Vps45, acting as a linker molecule between Rab and SM protein on early endosomes.[86] Vps33 is mainly studied in yeast where it functions in homotypic fusion of vacuoles. As mentioned above, unlike other SM proteins Vps33p is part of a multiprotein complex (HOPS or Vps/C) that interacts both with Rabs and SNAREs. Clearly, more work is needed before the still enigmatic role of these important proteins in membrane fusion is understood.

Late Steps in the Fusion Pathway and Fusion Catalysis

As discussed above, the SNAREs are the best candidates for fusion catalysts, and this view is supported by a large body of evidence. However, primarily studies on the homotypic

fusion of yeast vacuoles (the "last" fusion step in the endocytotic pathway) provided evidence that additional reactions are required downstream of the SNAREs before fusion. Using primarily the effects of inhibitors (such as antibodies) on the kinetics of in vitro fusion of vacuoles as argument, SNAREs pairing was primarily assigned to the docking reaction, and deemed expendable for the subsequent steps leading to fusion.[87] Events that were shown by these approaches to occur downstream of SNAREs include the action of a protein phosphatase, the release of calcium from vacuolar stores, and finally the "trans"-complexation of a certain type of the Vo-subunit of the vacuolar proton ATPase that is thought to be activated by calmodulin and then form a proteinaceous fusion pore.[22,26,88,89]

Docking causes a release of calcium from the vacuole.[90] In fact, many intracellular fusion reactions are dependent on the local release of calcium (including homotypic fusion of mammalian early endosomes or of ER-membranes). These fusions are completely blocked by fast calcium chelators, which can be overcome by readding free calcium. More recently, it was reported that SNAREpairing in "trans" during vacuole docking is directly responsible for the Ca^{2+} release.[91]

The target of the released calcium in the fusion pathway is calmodulin that selectively binds to the vacuolar membrane in a Ca^{2+}-dependent manner. The Ca^{2+}-calmodulin receptor on the membrane was subsequently identified as the Vo domain of the vacuolar proton ATPase.[88] This enzyme is conserved from archebacteria to higher eukaryotes and is responsible for the acidification of the lumen of the entire intracellular endomembrane system. Structurally, it has many similarities with the mitochondrial FoF1-ATPase but it is not capable of synthesizing ATP. Recently, it has been shown that the V-ATPase also has a head and a stalk domain that rotate with respect to the membrane-embedded proteolipid ring in the membrane, made up of Vo subunits.[92] The discovery that calmodulin appears to mediate its activation of vacuolar fusion via binding to Vo, and that this activation occurred downstream of SNARE involvement, seriously challenged the view that SNAREs are fusion catalysts. Rather, an alternative model was proposed according to which two proteolipid rings, arranged in "trans", would form a connexon-like fusion pore that would enlarge during fusion.[93]

Presently, it cannot yet be decided which of the two mutually exclusive models for the fusion mechanism is correct, although admittedly we have a hard time understanding that the conserved Vo subunit of a highly conserved proton pump shall have a "second life" (also referred to as "moonlighting") as a fusion pore. Recently, however, it has been shown that the block exerted by calcium chelators on yeast vacuole fusion can be completely rescued by adding the recombinant SNARE Vam7p.[94] This SNARE that operates as Qc-SNARE in vacuole fusion belongs to a small subgroup of SNAREs that do not possess a membrane anchor. Rather, Vam7p cycles off and on the membrane,[95] probably being aided by its phox-homology (PX) domain that selectively binds to phosphoinositol-3-phosphate.[96] The fact that Vam7p bypasses the chelator block seriously challenges the notion that the calcium-calmodulin system operates downstream of SNAREs, thus invalidating the hitherto strongest argument against the role of SNAREs as fusion catalysts in this fusion step.

Concluding Remarks

Despite major progress in identifying proteins involved in fusion reactions of the endocytic pathway, we just are beginning to understand some of the underlying molecular steps and the principles that govern these reactions. The advances in imaging fusion in live cells have shown that membrane traffic is much more dynamic than previously thought, with myriads of vesicles and cisternae being constantly on the move and continuously splitting and fusing. Despite the emerging molecular complexity these reactions must be robust and adaptable. It is becoming apparent that docking and fusion steps involve assemblies of macromolecules that are not stable but rather assembled on demand and dissociate once the task is completed. Furthermore, these molecular machines appear to represent "dirty" nanostructures that do not have a fixed stoichiometry and that appear to possess a high degree of redundancy, thus being able to afford even

major changes in the availability of individual components. While common principles are emerging including the stereotype action of the fusion catalysts, a lot needs to be learned about many of the other steps of the reaction cascade such as the initial signalling events and the layers of regulation that control fusion.

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CHAPTER 6

Clathrin Adaptor Proteins in Cargo Endocytosis

Linton M. Traub*

Abstract

Eukaryotic cells continuously remodel the protein and lipid composition of the plasma membrane in response to the extracellular milieu. Membrane retrieval typically involves inward budding of small bilayer-encapsulated vesicles that shuttle protein and lipid from the surface to internal endosomal elements. Clathrin-mediated endocytosis is a dominant pathway for internalization in many cell types, and a range of dedicated signals are used to ensure selective sorting in this pathway. Evidence suggests that the clathrin coat utilizes a diverse collection of clathrin-associated sorting proteins (CLASPs) to ensure the efficient and noncompetitive concentration of a wide variety of sorting signals within transport vesicles forming at the cell surface.

Introduction

The limiting membrane of eukaryotic cells represents the primary interface with the exterior environment. It performs a vital barrier function that, by tightly controlling the entry of molecules into the cell interior, contributes to long-term cell survival. The plasma membrane is also a platform for first detecting and then responding to extracellular signals, ions and nutrients, as well as potential pathogens. This is because a large array of receptors, ion channels, pumps and transporter proteins are positioned at the cell surface of a typical eukaryotic cell. Some receptors (mostly handling nutrient uptake or protein delivery) flux constantly through the plasma membrane while others, primarily involved in signal transduction, only exit the cell surface en masse following ligand stimulation. The process of regulated removal of certain receptors from the surface is perhaps best understood from the point of development,[1] where failure to quantitatively clear a particular receptor(s) from the plasma membrane can ultimately result in inappropriate cell fate determination due to erroneous cellular responses to local morphogens or growth factors.[2,3]

Sorting Signals for Selective Internalization

In principle, internalization of transmembrane proteins (along with bound ligands) could be either an active or a passive process. There are certainly examples of proteins being retained at the plasma membrane through stabilizing interactions with lipids,[4] lipid rafts,[5] or other proteinaceous components, like scaffolding proteins with PDZ domains for example.[6] Yet, it is generally accepted that endocytic uptake is governed by a positive signal;[7] in other words, there

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Endosomes, edited by Ivan Dikic.

is a process of preferential sorting of select transmembrane proteins for enrichment within forming carrier vesicles. This is because many proteins/receptors stagnate at the cell surface, redistributing into a diffuse pattern over the plasma membrane, when sorting signals within the cytosolic domain are inactivated either by inherited defects or directed mutation(s), or if the cytosolic region is simply truncated.[8,9] Indeed, numerous, structurally discrete sorting signals that specify rapid internalization are now known[7] (Table 1).

Clathrin-Mediated Endocytosis

There are several ways in which preferential entry of transmembrane proteins and bound ligands into the cytoplasm can occur. These include the budding of small (~60-100 nm) membrane bound vesicles in caveolin-dependent,[10] clathrin-dependent,[11,12] and uncoated caveolin-independent endocytosis,[13,14] as well as through larger forms of membrane internalization vehicle, as in macropinocytosis and phagocytosis.[12] Of these, probably the best characterized at the mechanistic level is clathrin-mediated endocytosis. Clathrin-coated buds and clathrin-coated vesicles were originally discovered in thin-section electron micrographs on the basis of a highly characteristic bristle-like matrix deposited on the cytosolic aspect of these structures[15] (see Fig. 1A). The unusual membrane invaginations also displayed striking concentration of electron dense material within the lumen, located opposite to the bristle coat.[15] These landmark morphological findings accurately mirror the two principal functional activities of clathrin-coated vesicles (and coat-dependent sorting in general); assembly of a polymeric, cytosol-oriented coat while simultaneously gathering select cargo molecules into the invaginating coated vesicle.

Table 1. Clathrin-dependent endocytic sorting signals

Signal Type	Examples		Recognition Protein/Domain	References
	Sequence	Protein		
YXXØ[a]	**YTRF**	transferrin receptor	AP-2, μ2 subunit	7, 19
	YSKV	CI[b]-MPR		
	YRGV	CD[c]-MPR		
	YQRL	TGN38/46		
	YQTI	LAMP-1		
	YATL	LRP1		
[DE]XXXL[LI]	**ERAPLI**	LIMP-II	AP-1, γ/σ1 hemicomplex	7, 47
	DKQTLL	CD3-γ		
	EKQPLL	tyrosinase	AP-2, α/σ2 hemicomplex?	
	DQRDLI	Ii		
[FY]XNPXY	**FDNPVY**	LDL receptor	ARH, Dab2,Numb PTB domain β-arrestin?	7, 19
	FTNPVY	LRP1		
	FENPMY	megalin		
	YTNPAF	Sanpodo		
Phosphorylation	–	GPCRs	β-arrestin 1/2	19, 64
Ubiquitin	–	EGFR	epsin, eps15 UIM[e] domain?	102, 103
		Notch		
		Delta		
		ENaC[d]		

[a] Consensus sequences indicated in single letter amino acid notation using PROSITE syntax. Ø indicates a bulky hydrophobic amino acid; Leu, Met, Ile, Phe, Val. [b] CI-MPR is cation-independent mannose 6-phosphate receptor. [c] CD-MPR is cation-dependent mannose 6-phosphate receptor. [d] ENaC is epithelial sodium channel. [e] UIM is ubiquitin interaction motif.

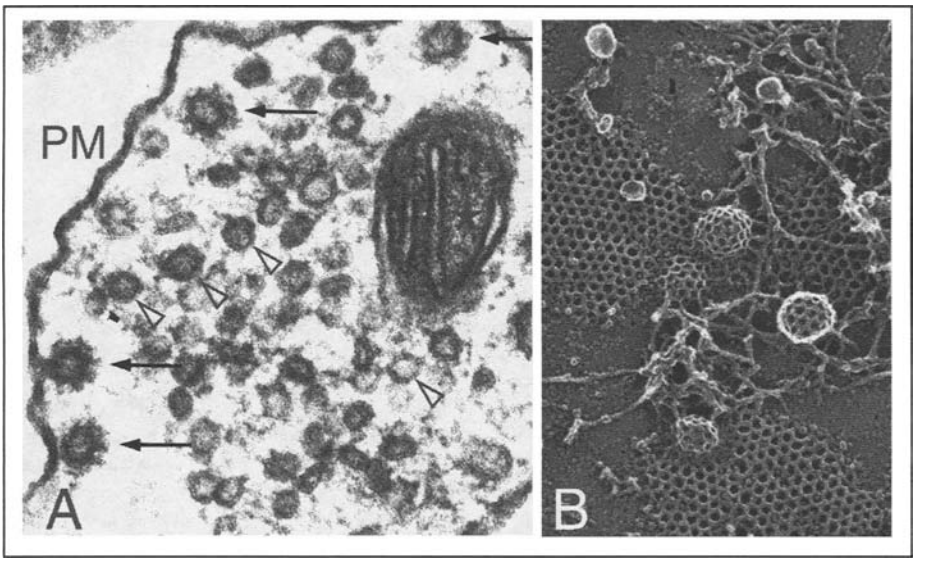

Figure 1. Clathrin-coat morphology. A) Thin-section electron micrograph of a purified rat brain nerve ending (synaptosome) revealing the characteristic cytosol-oriented 'bristle' matrix (arrows) enveloping several clathrin-coated vesicles budding from the presynaptic plasma membrane (PM). At the nerve ending, coated vesicles retrieve synaptic vesicle membrane components to replenish the pool of vesicles (arrowheads) required for sustained neurotransmission. A mitochondrion (asterisk) is positioned in close proximity to the synaptic region. B) Freeze-etch electron micrograph of the cytoplasmic face of the ventral plasma membrane of a cultured cell revealing the typical polyhedral clathrin lattice. The progressive invagination of coated membrane is clearly seen in different intermediates of the budding process. This image was graciously provided by John Heuser.

The 200 Å bristle-like structures radiating off clathrin-coated membranes correspond to the regular coat assemblage. The major constituent is, of course, clathrin, a tri-legged protein complex composed of three 192-kDa heavy chains and three ~25-kDa regulatory light chains, one bound to each heavy chain[16] (Fig. 2). Clathrin trimers can self-assemble onto spherical polyhedral assemblies in vitro, which are highly reminiscent of coated vesicles in vivo. It is the geometric nature of the triskelion that dictates the formation of the hexagon/pentagon-containing lattice, the most recognizable feature of the clathrin coat (Fig. 1B). The orientation and rigidity of the fibrous heavy chains imparts an inherent sidedness to the triskelion; when assembled upon biological membranes, the amino-terminal region of the heavy chain, which folds into a 7-bladed β-propeller structure,[17] is oriented closest to the bilayer. The carboxyl termini, which bundle together at the central vertex in a helical tripod arrangement, are positioned furthest from the membrane surface.[16] However, clathrin does not have the capacity to associate with phospholipid bilayers directly. Instead, adaptor proteins couple the clathrin lattice with the underlying membrane by synchronously binding to the clathrin terminal domain, to phospholipids, and to cargo sorting signals.

The AP-2 Adaptor Complex

A second major protein constituent of the endocytic clathrin coat is the AP-2 adaptor,[18] composed of four distinct subunits; two large ~100-kDa subunits (α and β2), a 50-kDa medium subunit (μ2), and a 17-kDa small subunit (σ2).[19] The functional complex has a characteristic architecture of two small appendages projecting off a larger globular core through

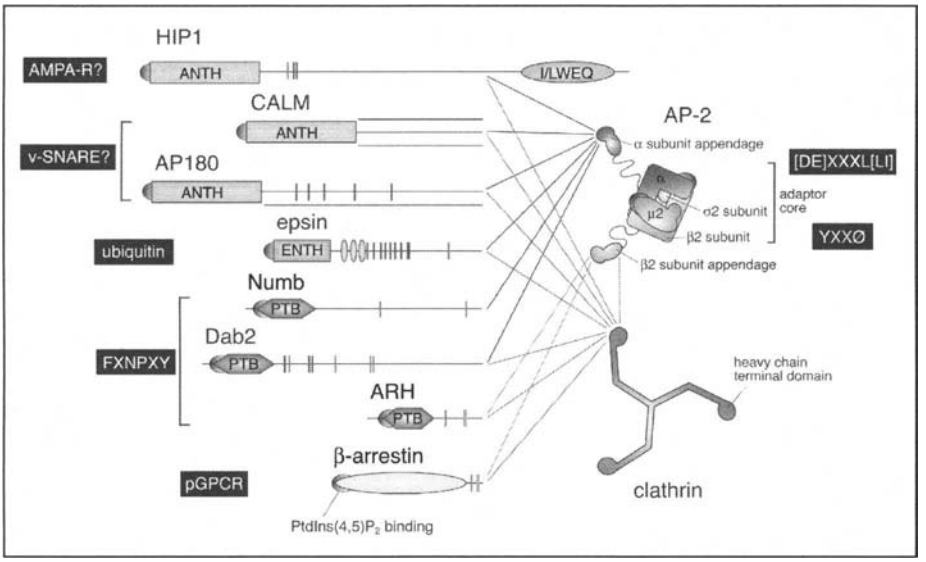

Figure 2. The clathrin–AP-2–CLASP interaction network. Schematic depiction of the protein–protein interactions between clathrin, AP-2 and selected CLASPs is shown. Images are modeled on the known molecular structures of the coat components, but are not drawn to scale. The clathrin terminal domain and the AP-2 appendages serve as platforms to coordinate the protein–protein interaction networks established during coat assembly. The location of interaction motifs used to engage these binding platforms are indicated by color-coded vertical lines. In AP180 and CALM, where the interaction motifs have not been defined precisely, interaction is generally signified by a horizontal line. The location of the three tandem UIMs in epsin 1 (ovals) is shown. pGPCR indicates phosphorylated (ligand-activated) G protein-coupled receptor, while I/LWEQ is a modular F-actin binding fold that is also conserved in the S. cerevisiae HIP1 orthologue Sla2p.

protease-sensitive stalks of apparently variable length.[20] Structural studies show the core is a heterotetramer comprised of the amino-terminal trunk regions of the large chains complexed with the smaller subunits[21] (see Fig. 2). The two appendages correspond to the independently folded carboxy-terminal segments of the large α and β2 subunits,[22-24] each attached to the respective trunk through an intrinsically unstructured intervening polypeptide hinge (for a comprehensive review of adaptor structure, see ref. 19). A five residue interaction motif (LLNLD; termed the clathrin box) is positioned within the pliable disordered hinge of the β2 subunit[25] and this binds directly to the clathrin terminal domain β-propeller.[26]

The AP-2 core complex seems to oversee coat operations close to the membrane. A patch of basic amino acids near the amino terminus of the α subunit binds to phosphoinositides, specifically phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$).[21,27,28] A second $PtdIns(4,5)P_2$-contact site is located on the μ2 subunit but, in the resting (cytosolic) state, the two chains are not positioned appropriately to allow simultaneous lipid binding. Importantly, the μ2 subunit also binds to a common class of sorting signal, the tyrosine-based YXXØ motif (where X is any amino acid and Ø represents a residue with a bulky hydrophobic side chain), found for example in the transferrin receptor, the mannose 6-phosphate receptors and the low density lipoprotein (LDL) receptor-related protein 1 (LRP1)[7] (Table 1). Classical models for clathrin-based sorting do not accommodate phospholipid support, and posit simply that coat assembly proceeds via the concerted linking of cargo with clathrin through the bifunctional AP-2 adaptor to fabricate a polymeric coat that progressively invaginates before budding into the cytoplasm. The final scission step is a complex process, handled by the large GTPase dynamin

and several accessory proteins such as amphiphysin, endophilin and sorting nexin 9.[29-31] Upon release of the transport vesicle, rapid, ATP-dependent uncoating allows both AP-2 and clathrin to reenter a soluble pool to initiate new rounds of coat assembly, while the vesicle is primed for fusion with acceptor endosomal elements.

But the structure of the AP-2 core reveals that in the basal state the surface of the μ2 subunit that recognizes the YXXØ sequence is clearly inaccessible.[21] This region of μ2 packs up against the adjacent β2 trunk and phosphorylation of μ2 at Thr156 is necessary to unleash the subunit for productive interactions with YXXØ sequences.[32,33] The repositioning of phosphorylated μ2 allows simultaneous binding of the YXXØ signal and PtdIns(4,5)P_2[21,34] strengthening the association of AP-2 with the plasma membrane. In addition, the catalytic activity of the kinase that phosphorylates Thr156, termed AAK1, is stimulated by assembled clathrin.[35,36] This suggests that AP-2 is focally activated to engage cargo as the coat assembles, possibly making cargo recognition a relatively late event. Indeed, μ2 subunit mutations that preclude YXXØ engagement do not prevent the proper deposition of AP-2 on the plasma membrane.[32,37] Nonetheless, cargo capture does appear to be a decisive step in the assembly process as live-cell imaging of clathrin dynamics in cultured cells shows that failure to concentrate cargo (transferrin) within an assembling lattice rapidly triggers catastrophic dissolution of the assemblage.[38]

Mutation or targeted gene disruption of individual AP-2 subunits is lethal in mice, *Drosophila melanogaster*[39] and *Caenorhabditis elegans*[40] (although not in *Saccharomyces cerevisiae*).[41,42] In cultured mammalian cells, post-transcriptional silencing of AP-2 α or μ2 subunit mRNA using small interfering RNA (siRNA) halts the internalization of the transferrin receptor[43-45] and reduces the number of morphologically discernable (bristle-like) clathrin coats at the surface more than tenfold.[44] These results clearly highlight the pivotal contribution AP-2 makes to clathrin-mediated endocytosis.

Diversity in Cargo Recognition Events

AP-2 is structurally and functionally homologous to the AP-1 and AP-3 heterotetramers that operate at the trans-Golgi network and on endosomes.[46] A nontyrosine sorting signal, the [DE]XXXL[LI]-type dileucine sequence (Table 1) binds to a hemicomplex of the γ1/σ1 or δ/σ3 subunits of AP-1 or AP-3, respectively, at a presently unknown location.[47] The [DE]XXXL[LI] sequence also acts as an efficient internalization motif[48] so it probable that AP-2 binds physically to this sorting signal as well. The capability of AP-2 to contact two discrete signals though separate subunits implies that individual coated vesicles can simultaneously carry multiple classes of cargo. In reality, endocytic clathrin coats do not only garner YXXØ- and [DE]XXXL[LI]-harboring proteins. Other receptors with tyrosine-based FXNPXY-type internalization sequences, like the LDL receptor, likewise cluster at assembled clathrin structures at the cell surface.[49-51] And still others, like the epidermal growth factor (EGF) receptor, also congregate in clathrin-coated regions following activation.[49,52] Although the EGF receptor contains a YXXØ signal (YRAL) within the cytosolic domain and binds directly to AP-2,[53] this segment is dispensable for internalization.[54] Accordingly, point mutations within the μ2 subunit that prevent YXXØ-sequence binding have little inhibitory effect on EGF receptor uptake.[37] In fact, ectopic receptor overexpression studies show unambiguously that saturating levels of FXNPXY or [DE]XXXL[LI]-containing proteins at the surface have negligible effect on the kinetics of transferrin or EGF receptor internalization and *vice versa*.[48,55,56] And each receptor type saturates the endocytic machinery at different surface densities.[56] One explanation for these general findings could be that the internalization of each cargo type is overseen by distinct sets of clathrin-coated vesicle with nonoverlapping cargo selectivity. Ultrastructural analysis of surface clathrin-coated regions argue strongly against this notion. In electron micrographs, receptors for transferrin, LDL, EGF and insulin and are clearly colocalized within individual coated profiles;[49,57] each clathrin-coated vesicle contains numerous types of cargo utilizing different sorting signals which, moments later, are found in common early endosomes.[58] Good coincidence of the transferrin and LDL receptor in individual punctate

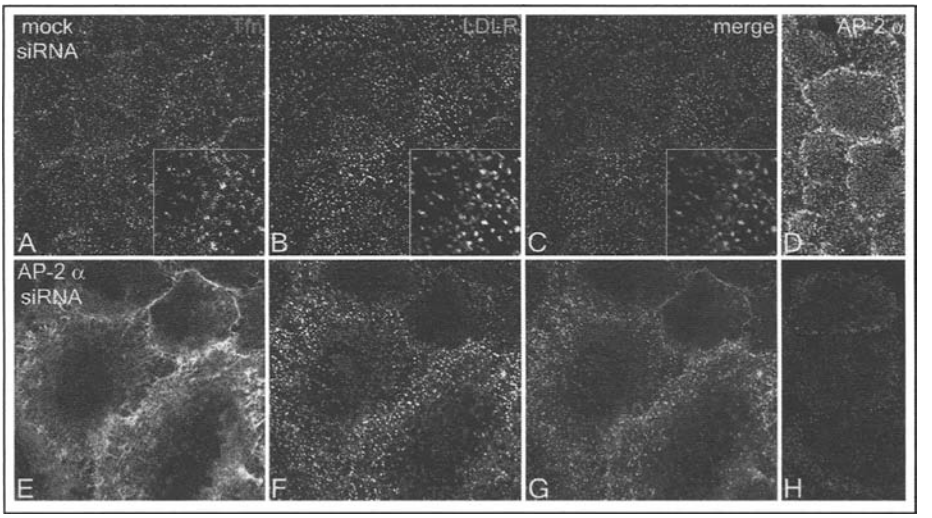

Figure 3. AP-2 does not regulate the sorting of all clathrin-dependent cargo. Single confocal optical sections of confluent HeLa cell monolayers treated either with mock (A-D) or AP-2 α subunit siRNA (E-H) and then double-labeled with fluorescent transferrin (A and E, red) and anti-LDL receptor antibodies (B and F, green), or stained with anti-AP-2 α subunit antibodies (D and H). In untreated cells, the transferrin and LDL receptors colocalize but after AP-2 silencing, only the transferrin receptor undergoes massive redistribution at the cell surface. Insets in A, B, and C show a magnified region to better judge the degree of colocalization between transferrin and the LDL receptor.

(clathrin-containing) sites on the plasma membrane is also seen by light microscopy (Fig. 3A-D). But, while RNAi silencing of the AP-2 α subunit (Fig. 3H) causes the transferrin receptor to diffuse over the cell surface (Fig. 3E), the LDL receptor, strikingly, remains concentrated in punctate zones (Fig. 3F) and internalizes rather efficiently, even in the absence of AP-2.[44] Entry of the LDL receptor is still clathrin dependent under these conditions because siRNA-mediated knock down of clathrin prevents the internalization of both transferrin and LDL receptors.[44] This capability of receptors lacking a YXXØ -or [DE]XXXL[LI]-type internalization signal to enter the cell in the absence of functional AP-2 suggests that alternate adaptor proteins must regulate the internalization of some classes of cargo and these might correspond to the differentially saturable connectors revealed in the overexpression studies.[48,56]

Clathrin-Associated Sorting Proteins (CLASPs), the Alternate Adaptors

Early clues to the nature of putative alternate adaptors came from studies on the seven-transmembrane-spanning G protein-coupled receptors (GPCRs). GPCRs are the most extensive superfamily of signaling receptors in eukaryotes and many, but not all, of these proteins are quickly downregulated by clathrin-mediated endocytosis following agonist-induced activation.[59-61] Ligand binding prompts phosphorylation of the cytosolic region of most GPCRs. This reversible modification acts as a trigger for one of two ubiquitous arrestin family members (β-arrestin 1 or β-arrestin 2) to translocate onto the activated GPCR.[59-61] The β-arrestins desensitize the receptor by uncoupling signaling events, but also direct the phosphorylated GPCR to clathrin-coated structures for prompt internalization. The capacity of β-arrestins to promote GPCR endocytosis depends upon four fundamental properties of these adaptor proteins: the ability to bind physically to the GPCR, to PtdIns(4,5)P_2, to AP-2 and to clathrin.[62-64] Interaction motifs

arrayed tandemly within the carboxy-terminal segment of the β-arrestins specify direct interactions with the clathrin heavy chain β-propeller[26,65,66] and with the appendage of the AP-2 β2 subunit.[63] All four interactions are required for efficient GPCR endocytosis, and β-arrestins work by melding the GPCR with preexisting clathrin coats by synchronously engaging both.[67] These are essentially the properties of the archetypical adaptor, AP-2, but it is the β-arrestins, and not AP-2 directly, that are charged with overseeing the internalization of the largest family of receptors known. There is also evidence that β-arrestin 2 handles the uptake of nonclassical seven-transmembrane-spanning smoothened[68] and frizzled[69] receptors that operate in the Wnt and Hedgehog signaling pathways.[1] Also, β-arrestin 2 apparently participates in the internalization of transforming growth factor β (TGFβ) by binding to the type III TGFβ receptor (also termed betaglycan) in a phosphorylation-sensitive manner.[70] And finally, β-arrestin nullizygous mice fed a high fat diet display defective lipoprotein metabolism and it appears that β-arrestin 2 may also modulate constitutive LDL receptor endocytosis by binding to the cytosolic domain of the receptor in a phosphorylation-dependent fashion.[71]

The cargo selective properties of the β-arrestins make them the founding members of the CLASP family. But certain other so-called endocytic 'accessory' proteins[72] also display the important binding properties typical of the β-arrestins (and AP-2).[73] These presumptive CLASPs bind to PtdIns(4,5)P_2, albeit using different modular lipid-binding folds, bind to the clathrin β-propeller domain, and all can engage the appendages of the large AP-2 adaptor subunits[19,46,73] (Fig. 2). CLASPs appear to operate by contributing to the efficiency of clathrin lattice assembly while simultaneously expanding the sorting repertoire of the forming coat.[46,74] Currently, the experimental evidence for a set of dedicated, phosphotyrosine-binding (PTB) domain-containing CLASPs governing LDL receptor internalization is most compelling.

PTB Domain CLASPs and FXNPXY Signal Endocytosis

The PTB acronym is actually a misnomer. Although the domain was originally identified as a non-SH2 phosphotyrosine binding module,[75] it is clear that tyrosine phosphorylation is not a prerequisite for productive binding of the canonical PTB binding-partner sequence [FY]XNPXY.[76] A form of this sequence positioned within the cytosolic domain of the LDL receptor (FDNPVY), in its nonphosphorylated form, was actually the first internalization signal ever discovered.[77] Two PTB domain CLASPs, designated Disabled-2 (Dab2) and the autosomal recessive hypercholesterolemia (ARH) protein, appear to decode the FXNPXY internalization signal in the LDL and related receptors. Inherited mutations or targeted gene disruption of these proteins cause obvious pathological phenotypes.[78-80] For ARH, individuals with inactivating mutations in both *ARH* alleles have early onset hypercholesterolemia that is very difficult to distinguish from that typical of patients entirely lacking LDL receptor function.[78,81-86] Thus, these unfortunate individuals present with a disease that closely mirrors a complete failure to bind and clear circulating LDL particles. In full agreement, $ARH^{-/-}$ mice show dramatic accumulation of LDL receptors at the sinusoidal plasma membrane of hepatocytes, the cell type responsible for clearing the bulk of circulating LDL from the plasma.[87]

In a similar fashion, Dab2 nullizygous mice display a proteinuria because of ineffective sorting of an LDL superfamily receptor termed megalin at the apical surface of renal proximal tubules.[79,88] Megalin is a scavenger receptor that retrieves considerable amounts of albumin, vitamin- and lipid-binding proteins from the glomerular filtrate to prevent protein excretion in the urine;[79] the $Dab2^{-/-}$ phenotype is actually a milder version of that seen upon megalin gene disruption.[89] ARH and Dab2 use a single folded PTB domain to bind the FXNPXY sequence and PtdIns(4,5)P_2 noncompetitively.[51,90-92] This promotes cargo recognition, while binding to the clathrin coat is controlled by the carboxy-terminal segment in both proteins.[51,90,93,94] Just like the β-arrestins, ARH and Dab2 have tandemly arrayed interaction sequences that promote associations with the AP-2 appendages and the clathrin β-propeller (Fig. 2). That these proteins can contribute to clathrin coat assembly is evidenced by a decrease in detectable clathrin-coated vesicles at the apical pole of Dab2-null proximal tubules.[79]

Secondary structure predictions suggest that outside of the modular amino-terminal PTB domain, these proteins are largely unstructured. The reason for this may be that intrinsically disordered tracts of polypeptide are highly mobile and pliable, which allows a large 'capture radius' for binding partners. This property could draw receptor–CLASP complexes into a pre-existing clathrin structure rather efficiently. A gross absence of secondary structural elements is also consistent with what is known about the mode of engagement of various interaction motifs with the clathrin β-propeller and the AP-2 appendages.[26,95-97] In fact, extended regions of unstructured random-coil polypeptide turns out to be an unexpected characteristic of the CLASP group of endocytic components.[73,98]

Another very informative example of the endocytic action of a PTB-domain CLASP, termed Numb, is in the biogenesis of retro-orbital neurosensory bristles in *Drosophila*.[99] The mature sensory bristle is composed of four cell types; hair, socket, neuron and sheath cells. The four all arise from a single sensory organ precursor (SOP) cell by two rounds of asymmetric cell division.[3,100] During SOP cell mitosis, Numb distributes asymmetrically in the mother cell so as to selectively partition into only one of the two daughter cells.[100] Mutant *numb* alleles lead to cell fate transformations, resulting in only four socket cells being formed.[3,100] Mutations that disrupt the activity of the appendage of the AP-2 α subunit, to which Numb binds directly, generate a phenotype equivalent to *numb* mutants.[3] The simplest model for Numb activity is that asymmetric Numb–AP-2 association during cell division results in one of the two siblings having a considerably higher Numb/AP-2 complement, which then drives the removal of the transmembrane Notch receptor from the surface in this cells. This, then, changes the fate of the cell compared to the other daughter.[3,99] Numb binds to Notch, yet there are no obvious differences in Notch expression level between the two cells that ultimately become quite different cell types. Instead, a Notch-binding accessory protein termed Sanpodo, seems to be the surface component downregulated in Numb-enriched daughter cells.[101] Sanpodo is a tetraspannin membrane protein with a variant [YF]XNPXY sequence (YTNPAF) in the cytosolic portion and Numb-mediated clearance of Sanpodo from the surface is suggested to compromise Notch signaling, leading to a different cell fate.[101]

ENTH/ANTH-Domain Containing CLASPs

Posttranslational conjugation of ubiquitin is another important method of generating sorting signals, as covered in several other chapters of this book (see chapters by Puertollano, Pattni and Stenmark, and Gur et al). A discussion of the nature and generation of this internalization signal is beyond the scope of this chapter, but the epsin family of CLASPs (together with eps15, an epsin binding partner) are good candidates for recognizing ubiquitinated cargo at the cell surface.[102,103] Again, the conserved modular architecture and binding properties of epsin are fully compatible with a role as a cargo-selective adaptor. The epsin N-terminal homology (ENTH) domain binds directly to PtdIns(4,5)P_2,[104] while two or three ubiquitin-interaction motifs (UIMs) enable the protein to bind physically to ubiquitin[105] (Fig. 2). The UIMs are located between the ENTH domain and the unstructured carboxy-terminal region, housing standard interaction motifs that enable epsin to bind both clathrin and the AP-2 appendages[106-108] (Fig. 2).

In *Drosophila*, while a PTB-domain CLASP (Numb) attenuates Notch receptor signal transduction in certain SOP progeny, an ENTH-domain CLASP (epsin) actively promotes Notch signaling.[109-112] Notch is activated in a signal-receiving cell by engaging the transmembrane ligand Delta, presented at the surface of an adjacent signal-sending cell.[113] The Notch transcriptional response is preceded by two ordered, ligand-induced cleavage events that sever Notch on both sides of the plasma membrane allowing the intracellular domain to translocate to the nucleus.[113] It appears that the second proteolytic event that liberates the Notch intracellular domain requires prior endocytosis of the extracellular region of Notch, bound to Delta, into the signal-sending cell. There are strong genetic interactions between Delta, two E3 ubiquitin ligases (Neuralized and Mind bomb), and the fly epsin, termed Liquid facets.[109-112] Current

evidence strongly suggests that the E3-mediated ubiquitination of Delta that allows Liquid facets to internalize the Delta/Notch complex is obligatory for Notch signaling. The pivotal role of Liquid facets in promoting Notch signaling is underscored by the participation of the deubiquitinating enzyme Fat facets. Fat facets appears to operate by buffering the intracellular epsin concentration by salvaging proteasome destined, polyubiquitinated Liquid facets by deubiquitinating the protein.[114] In fact, expression of a single extra copy of Liquid facets abolishes the requirement for Fat facets in the developing compound eye; there is no eye phenotype in Fat facets-null flies expressing an extra Liquid facets gene.[2] The *Drosophila* data suggest that during development, the intracellular abundance of epsin is critical, presumably to effect the timely endocytosis of certain membrane proteins/receptors thereby facilitating appropriate cell fate determination.[2,110,113] This is in full accord with the concept of epsin functioning as a CLASP. Likewise, in mammals, another ENTH domain protein, huntingtin interacting protein 1 (HIP1) displays a variation on the general CLASP architecture (Fig. 2), and may manage the internalization of AMPA receptors in the central nervous system.[115]

Finally, the structure of the AP180 N-terminal homology (ANTH) domain is highly related to the ENTH module and binds to PtdIns(4,5)P_2, although by a different molecular mechanism.[19] AP180 and the nonneuronal orthologue CALM both bind clathrin and AP-2. As for function, UNC-11, the *C. elegans* AP180, is abundantly expressed in neurons and analysis of the effect of unc-11 mutants suggests that this protein regulates the retrieval of the v-SNARE synaptobrevin from the presynaptic plasma membrane.[116]

Conclusions and Perspective

The identification of cargo selective CLASPs allows several previously unexplained findings now to be resolved. General collaboration between AP-2 and CLASPs can adequately explain how PtdIns(4,5)P_2 within the cytosolic leaflet of the plasma membrane can act as a compartmental cue to direct the assembly of endocytic clathrin coats. The overall architectural similarity of the CLASP family is striking (Fig. 2), and the capability of numerous CLASPs to bind physically to both AP-2 and clathrin could account for the very rapid assembly of coated vesicles and the swift internalization of a diverse array of transmembrane proteins from the cell surface. Perhaps most importantly, the activity of these proteins can explain how internalization of select transmembrane proteins can continue in cells essentially lacking functional AP-2.[43-45]

Intriguingly, vesicle coats that operate at other sorting stations within the cell (the COPI and COPII coats, for example) do not appear to have diversified cargo capture operations to involve a host of CLASP-like components.[73,117] Thus a legitimate question is why there are so many endocytic CLASPs (and we may not yet have the complete list of cargo specific components operating at the cell surface). As discussed above, in *Drosophila*, the Notch pathway utilizes both PTB- (Numb) and ENTH- (Liquid facets) domain CLASPs to fine-tune signaling activity. But Notch/Sanpodo and the ligand Delta are endocytosed in different cells, the signal-receiving and signal-sending cell, respectively. So one obvious possibility is that the complexity of the endocytic CLASP network (Fig. 2) is substantially reduced in individual cell types. In other words, perhaps not all coated structures contain a full complement of CLASPs. This seems unlikely, however, because in SOP cell progeny for example, the daughter cell utilizing Numb to suppress intracellular Notch signal transduction is simultaneously using Liquid facets to promote effective Notch signaling in the adjacent sister cell. In fact, the vast majority of the core CLASP members are found both in brain and in cultured lines, like HeLa cells and fibroblasts. It therefore seems more probable that numerous different CLASPs populate a single clathrin structure assembling at the surface. The synchronous operation of a diverse group of CLASPs has the clear advantage of equipping individual clathrin coats with the capability of capturing an extended range of cargo molecules at the cell surface.

But even with the incorporation of CLASP activity into models for clathrin-based sorting, our general understanding of the molecular events that underpin coat assembly is still rather rudimentary. A major challenge is now to begin to understand the temporal chronology and

regulation of the numerous protein–protein interactions necessary for the successful fabrication of a clathrin-coated vesicle. While CLASPs clearly bind to the AP-2 appendages, there exists a diverse set of appendage binding sequences and two spatially separate contact sites upon each appendage.[19,73,118] Unfortunately, the static representation of dense network connectivity (Fig. 2) cannot adequately explain the assembly and cargo selection process chronologically; we do not understand the flow and processing of information. For instance, the biologic advantage of one CLASP displaying a particular set of appendage-binding sequences and not another is not at all clear. Presumably these sequence dictate the temporal behavior and possibly the rank of a particular CLASP, but there is a paucity of functional information on the individual contributions of these interactions to the coupled process of sorting and budding. It seems certain that the application of a wide array of experimental approaches, including live-cell imaging,[38,119] RNAi and gene replacement, time-resolved proteomics[120] and, perhaps most importantly, computational analysis and network theory modeling[121] will be necessary to unlock all the secrets of this highly efficient and sophisticated vesicular shuttle.

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CHAPTER 7

Protein Sorting in Endosomes

Krupa Pattni and Harald Stenmark*

Abstract

Molecules delivered to endosomes by endocytosis or biosynthetic trafficking can be either recycled to the cell surface, transported to lysosomes, or shunted retrogradely to the biosynthetic pathway. The distinct fates of different endosomal cargo molecules point to the existence of sorting machineries able to distinguish between cargoes. In this review we will highlight recent studies that are beginning to elucidate the endosomal sorting machineries that recognize different cargoes, as well as individual sorting signals that specify their destinations.

Introduction

Endocytosed or biosynthesized molecules that transit through endosomes have several possible destinations (see Fig. 1). They can be either sorted for recycling to the plasma membrane, anterograde trafficking to the degradative lysosomes or retrograde transport to the trans-Golgi network of the biosynthetic pathway. Since different endosomal cargo molecules have distinct itineraries, efficient sorting mechanisms must exist that recognize specific cargoes. Accumulating evidence suggests that all the above-mentioned trafficking routes out of endosomes rely on the recognition of specific cargo sorting determinants by distinct endosomal sorting machineries. Here, we will discuss emerging data that are beginning to shed light on the sorting determinants of endosomal cargo proteins and the machineries that recognize them.

Endosomes As Sorting Stations in Intracellular Membrane Trafficking

The organisation of the endocytic pathway is reviewed elsewhere in this book (Chapter 1). For the purpose of this review, we will distinguish between early endosomes (EEs), recycling endosomes (REs) and late endosomes (LEs). As outlined in Figure 1, recycling to the plasma membrane can either occur directly from the EEs or indirectly via the RE, in processes controlled by the small GTPases Rab4 and Rab11, respectively.[1] Typical examples of recycled membrane proteins include the receptors for transferrin and low-density lipoprotein.[2] In EEs, sorting towards the degradative pathway also takes place, as exemplified by ligand-activated growth factor receptors such as the epidermal growth factor (EGF) receptor.[3] Most membrane proteins destined for LEs and lysosomes are targeted into intraluminal vesicles that invaginate from the limiting membrane of the EE. Sorting to the trans-Golgi network (TGN) can occur from both EEs and LEs, most probably through different sorting machineries (see below).

The architecture of various types of endosomes reflects their specific purposes. For instance, the tubular morphology of REs and the "recycling" part of EEs ensures a high

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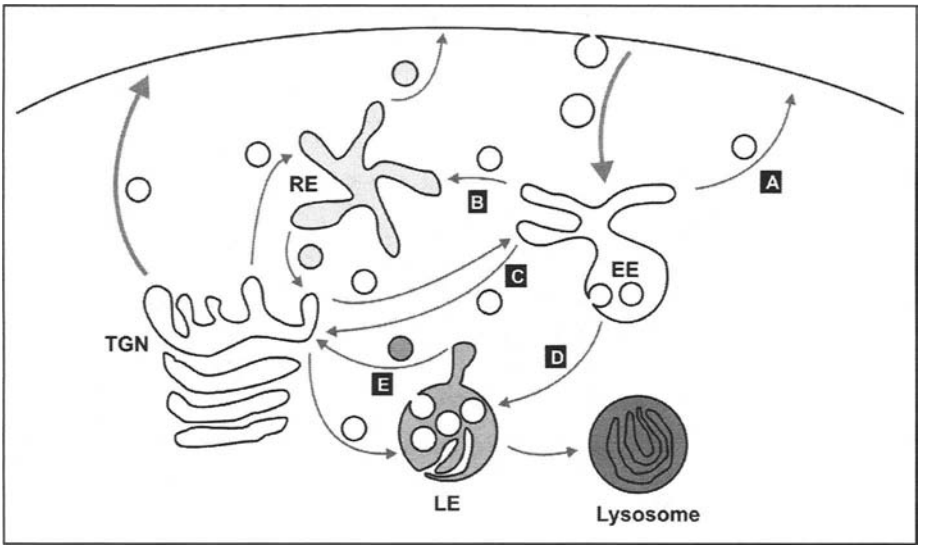

Figure 1. Protein sorting in the endocytic pathway of a nonpolarized cell. Upon endocytosis, cargo is transferred to the early endosome (EE). From here, cargo can be sorted for direct recycling to the plasma membrane (A), recycling via the recycling endosome (RE) (B), transport to the trans-Golgi network (TGN) (C) or transport to the late endosome (LE) (D). Sorting to the TGN can alternatively occur from the LE (E). Various trafficking steps are indicated by arrows whose line thickness reflects relative importance.

membrane-to-volume ratio. This favours an enrichment, and thus sorting, of endosomal membrane proteins with respect to soluble content, into transport carriers that leave the endosomal tubules.[2] In this way, small tubules and vesicles that bud from the REs and the tubular regions of the EEs are efficient vehicles for membrane proteins, mostly targeted for recycling to the plasma membrane. Another peculiar geometric feature of endosomes is the invagination of the cisternal part of the endosome membrane to form intraluminal vesicles (see Fig. 1). Since the membrane of such vesicles is more accessible to digestion by lysosomal enzymes than the limiting membrane of LEs (probably due to differences in lipid compositions and lower abundance of highly glycosylated membrane proteins), such vesicles are ideally suited as vehicles for membrane proteins destined for degradation.[4] The molecular machineries responsible for the formation of intraluminal vesicles, and for the sorting of membrane proteins into them, are beginning to emerge, and this will be discussed in the following sections (Table 1).

Sorting to the Recycling Route

The transferrin receptor (TfR) has served as a prototypic example of a recycling membrane protein. This receptor is constitutively endocytosed from clathrin-coated pits regardless of ligand binding. Upon reaching the EE, the TfR is very efficiently recycled to the plasma membrane. This recycling occurs both directly and via the RE.[5] The finding that a truncated TfR lacking the whole cytoplasmic tail recycles at the same rate as the wild-type receptor[6] initially led to the conclusion that endocytic recycling is signal independent.[7] However, more recent studies have revealed that multiple receptors, including the TfR, contain bona fide recycling determinants. The TfR has been found to contain two phenylalanine-based signals which, when mutated to alanine, slow down TfR trafficking from and to the RE.[8] These recycling sorting signals interact with ACAP1, a GTPase-activating protein for Arf6, which promotes cargo sorting to enhance TfR recycling.[8] In addition, ACAP1 interacts with cellubrevin, another recycling cargo

Table 1. Protein sorting in endosomes

Sorting Step (See Fig. 1)	Cargo (Example)	Sorting Signal	Sorting Machinery	Refs.
EE → PM (A)	TfR	?	Rab4, AP-1, Hrs, actinin-4, BERP, myosin-V	9,82
	β2-AR	Ser-411 phosphorylation by GRK5	EPB50, Hrs	12,13
	μ-opioid receptor	LENLEAE motif	Hrs	13
	D1 dopamine receptor	25-aa C-terminal sequence	?	16
	LHR	Palmitoylation	?	83
EE → RE → PM (B)	TfR	LF and RF motifs	Rab11, ACAP1	8
	Cellubrevin	?	Rab11, ACAP1	8
EE → TGN (C)	CI-M6PR	?	Retromer, EpsinR	61,68
	TGN38	SXYQRL motif	EpsinR	68,84
	Shiga toxin	(The receptor for Shiga toxin is the lipid Gb3)	Clathrin, EpsinR	68,69
EE → LE (D)	activated EGFR	Ubiquitination	Hrs, STAM, ESCRTs, GGA3	17,40,41, 85-87
	activated CXCR4	Ubiquitination	Hrs	79
	misfolded CFTR	Ubiquitination	Hrs, ESCRTs	88
	δ-opioid receptor	C-terminal α-helical region of cytosolic tail	GASP	52
	LAMP-1	C-terminal YQTI motif	AP-3	55,89
LE → TGN (E)	CD-M6PR	LL and FW motifs	Rab9, TIP47	90-92
	CI-M6PR	PPAPRPG motif	Rab9, TIP47	90,91
	Furin	SDSEED motif	?	93

The table refers to the sorting steps indicated in Figure 1 and provides examples of well-studied cargo molecules in mammalian cells as well as, when known, the signals and machineries that mediate their sorting. Note that some cargoes, including TfR and M6PR, can be sorted along at least two distinct pathways, and that the distinctions between pathways A and B have so far been incompletely defined for most cargoes. β2-AR: β2-adrenergic receptor; LHR: luteinizing hormone receptor; LAMP: lysosome-associated membrane protein; CD: cation-dependent; CI: cation-independent; CFTR: cystic fibrosis transmembrane conductance regulator; CXCR4: a chemochine receptor.

protein, but not with Lamp1, a cargo protein transported to lysosomes. ACAP1 may thus have a general function in endocytic recycling via the RE.

A recent study has shed light on how the TfR may be differentially recycled directly to the plasma membrane. A complex called CART (cytoskeleton-associated recycling or transport), consisting of hepatocyte growth factor regulated tyrosine kinase substrate (Hrs), actinin-4, brain-expressed RING-finger protein (BERP) and myosin-V, was found essential for fast recycling of TfRs. Disruption of this complex led to a slower recycling via the RE.[9] Thus, it is likely that ACAP1 sorts the TfR into the indirect recyling pathway.

How can these findings be reconciled with the efficient recycling of the truncated TfR?[6] One possibility is that the cytoplasmic tail of the TfR may contain endosomal retention signals in addition to recycling signals. Such signals, one consisting of an acidic cluster and one consisting of a di-leucine motif, have already been identified in insulin-regulated aminopeptidase, a slowly recycling membrane protein.[10] Even though these retention signals are reminiscent of

endocytic signals recognized by adaptor protein complexes, the specific machinery that recognises the endocytic retention signals has not yet been identified.

The first demonstrations of endocytic recycling signals have come from studies of G-protein-coupled receptors (GPCRs).[11] Most GPCRs are internalised in response to ligand binding, and many GPCRs display rapid recycling. GRK-5-mediated phosphorylation of serine-411 in the cytoplasmic tail of the β2-adrenergic receptor is required for efficient recycling of this GPCR. Sorting into the recycling pathway appears to be mediated by an interaction of the phosphorylated receptor with the PDZ domain of EBP50 (ezrin-radixin-moesin-binding phosphoprotein 50), a protein associated with the actin cytoskeleton.[12] Hrs, a constituent of the CART complex that mediates rapid recycling of TfRs, has recently been found essential for efficient recycling of the β2-adrenergic receptor as well as for recycling of another GPCR, the μ-opioid receptor. The N-terminal VHS-domain seems crucial for this atypical function of Hrs.[13] So far, the functional relationship between Hrs/CART and EBP50 in GPCR sorting has not been clarified.

While the β2-adrenergic and μ-opioid receptors appear to employ related mechanisms for their sequence-dependent recycling, other GPCRs probably use distinct mechanisms for their sorting into the recycling pathway. The lutropin receptor contains a 17-residue membrane-distal cytosolic signal that is necessary and sufficient for recycling of the endocytosed receptor.[14,15] In this receptor, specific leucine, cysteine, glycine and threonine residues have been implicated in the recycling function. Likewise, the D1 dopamine receptor contains a distinct 23-residue membrane-proximal sequence that mediates its recycling.[16] All the above-mentioned short sequences can be transplanted onto nonrecycling model receptors and mediate their recycling, suggesting that they interact directly with the sorting machineries that mediate endocytic recycling. So far, these machineries are not known, but the tools should now be available for their identification.

Sorting to the Degradative Pathway

In mammalian cells, ligand-bound (activated) growth factor receptors serve as prototypic examples of membrane proteins that are delivered to the degradative pathway after endocytosis (see chapter 9). As discussed in the following section, covalent attachment of mono-ubiquitin to one or several lysine residues in the cytoplasmic regions of a membrane protein represents an important sorting signal for the degradative pathway.[17,18]

Mono-Ubiquitin As a Degradative Sorting Signal

A number of membrane proteins are mono-ubiquitinated at the plasma membrane or on endosomes. This posttranslational modification has been shown to serve as an endocytosis signal in some cases, but the most general function of mono-ubiquitination in membrane traffic appears to be its role in endosomal sorting.[19] In fact, mono-ubiquitin serves as a dominant signal for degradative protein sorting, as illustrated by the fact that recombinant ubiquitin fusions of endocytic membrane proteins are efficiently targeted to lysosomes.[20,21] Even though a single mono-ubiquitin moiety may be sufficient for degradative protein sorting,[17,19,22] many membrane proteins, including growth factor receptors, are mono-ubiquitinated at multiple lysine residues (multi-ubiquitinated; see Fig. 2).[17,18] Mono- and multi-ubiquitination are mediated by the same sets of substrate-specific ubiquitin ligases, whose impaired function may be associated with diseases such as cancer (see Chapter 9). Recent work has shed light on the mechanisms of sorting of ubiquitinated membrane proteins. It is now clear that ubiquitinated cargo is recognised by a number of sorting components that contain specialised ubiquitin-binding domains (UBDs; see Fig. 2).[23]

Eight UBDs have been identified to date. These include the UIM (ubiquitin-interacting motif),[24] UBA (ubiquitin-associated domain),[25] UBC (ubiquitin-conjugating enzyme-like)/UEV (ubiquitin E2 variant),[26,27] CUE (Cue1-homologous),[28,29] GAT (GGA and TOM1),[32] GLUE,[33] PAZ (poly-ubiquitin-associated zinc finger),[30] and NZF (novel zinc finger)[31]

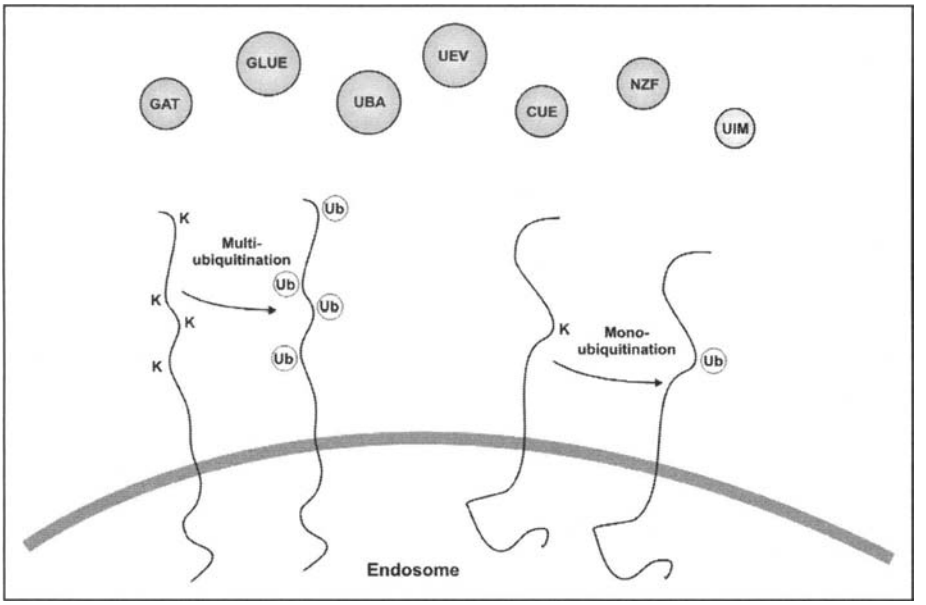

Figure 2. Mono- and multi-ubiquitin as sorting signals. Mono- and multi-ubiquitinations of endosomal membrane proteins, catalysed by substrate-specific E3 ubiquitin ligases, are illustrated schematically.[23] The mono- and multi-ubiquitinated proteins are recognised by the various UBDs indicated on top. The avidity for multi-ubiquitinated cargo is probably increased by multimerisation of UBD-containing proteins.

domains. With the exception of the PAZ domain, all the UBDs have been found within various subunits of endosomal sorting complexes (see Table 2). Even though these UBDs have diverse structural folds, they all contact the hydrophobic Ile-44 patch of ubiquitin. The affinity of this interaction is rather weak and is stronger for poly-ubiquitin chains than for mono-ubiquitin.[19] The affinity and specificity of the interaction to mono-ubiquitin is probably increased in vivo by additional interactions with the modified protein or formation of multimeric complexes of ubiquitin-binding proteins.[17]

Ubiquitin-Dependent Degradative Protein Sorting

Distinct UBD-containing proteins complexes are recruited to the plasma membrane and to endosomes. In the endosome membrane, the ubiquitinated membrane protein is recognised by a UBD-containing machinery that sorts it into intraluminal vesicles. The ubiquitin-binding endosomal protein Hrs is central to this machinery.[34] This protein is recruited to endosomal membranes through binding of its FYVE domain[35] to phosphatidylinositol 3-phosphate (PI3P), a phosphoinositide localized specifically to these membranes.[36,37] The C-terminus of Hrs binds clathrin,[38] and Hrs is found in a characteristic flat clathrin coat on EEs.[21,39] Hrs recognises ubiquitinated cargo via its UIM.[21] Although Hrs binds ubiquitin with low affinity, its avidity for multi-ubituitinated cargo may be increased by the fact that membrane-bound Hrs is complexed with two other ubiquitin-binding proteins, signal-transducing adaptor molecule (STAM) and Eps15 (see Table 2).[40] The function of the clathrin lattice could be to concentrate Hrs/STAM/Eps15 in restricted microdomains in order to increase sorting efficacy. Interestingly, another clathrin-binding protein, GGA3 (Golgi-localizing, gamma-adaptin ear domain homology, ARF-binding protein 3), has recently been found to interact with and sort ubiquitinated cargo for degradation.[41] The relationship between GGA3 and Hrs will thus be

Table 2. Ubiquitin-binding proteins involved in degradative endosomal sorting

Protein	Organism	Function	UBD	References
Rabex-5	Mammals	GEF for the GTPase Rab5, a regulator of trafficking through early endosomes	?	94
Vps9	Yeast	Homologue of Rabex-5	CUE	28,95
Eps15	Mammals	Endocytosis, endosomal sorting, in complex with Hrs and STAM on endosomes	UIM	40,96
Ede1	Yeast	Homologue of Eps15	UBA	97
GGA3	Mammals	TGN-/endosomal sorting, interacts with Tsg101	GAT	41,98
Gga1/Gga2	Yeast	Homologues of GGA3	GAT	99
Tom1	Mammals	Endosomal sorting, in complex with Tollip and Endofin	GAT	32,100
Tom1L1	Mammals	Endosomal sorting, interacts with Hrs and Tsg101	GAT	101
Hrs	Mammals	Endosomal sorting, in complex with STAM and Eps15, recruits ESCRT-I	UIM	21,40,45, 86,102,103
Vps27	Yeast	Homologue of Hrs	UIM	97,104
STAM	Mammals	Endosomal sorting, in complex with Hrs and Eps15	UIM,VHS*	40,105,106
Hse1	Yeast	Homologue of STAM	UIM	104,107
Tsg101	Mammals	Endosomal sorting, subunit of ESCRT-I	UEV	47,86
Vps23	Yeast	Homologue of Tsg101	UEV	26,49
Eap45	Mammals	Endosomal sorting, subunit of ESCRT-II	GLUE	33
Vps36	Yeast	Homologue of Eap45	NZF	43,108

Endosomal UBD-containing proteins from yeast and mammals are listed. UBD-containing proteins thought to function mainly in the TGN or at the plasma membrane have not been included. *In the case of mammalian STAM, a VHS (Vps27, Hrs, STAM) domain has been implicated in ubiquitin-binding in addition to the UIM.

interesting to examine. Hrs has a function that is conserved from yeast to man, and the sorting machinery immediately downstream of Hrs is also highly conserved.[42] Three endosomal sorting complexes required for transport (ESCRTs) were first identified in yeast and subsequently shown to be conserved in mammals.[26,43,44] Current evidence suggests that ESCRT-I is recruited to endosome membranes by a direct interaction with Hrs,[45,46] that ESCRT-II is recruited by binding to ESCRT-I,[43] and that ESCRT-III is recruited by ESCRT-II.[44] Both ESCRT-I and ESCRT-II contain ubiquitin-binding subunits (see Table 2), and it is conceivable that ubiquitinated cargo is transferred from Hrs to ESCRT-II via ESCRT-I (Fig. 3). Whereas ESCRT-I and ESCRT-II have defined biochemical compositions, and their partial crystal structures have been solved,[47-50] the composition of ESCRT-III is less well understood. This complex consists of two heterodimeric subcomplexes that assemble into large multimers on membranes (Fig. 3). It is thought that ESCRT-III multimerisation contributes to form inward invaginations and vesiculation of the endosome membrane, by a hitherto undefined mechanism.[44] The AAA-ATPase Vps4 mediates the disassembly of ESCRT-III complexes, thus allowing the same complexes to participate in multiple rounds of transport.[51]

Ubiquitin-Independent Degradative Sorting

Even though ubiquitin serves as a widespread sorting signal for the degradative pathway, there are also examples of nonubiquitinated membrane proteins that reach this pathway.[20,52]

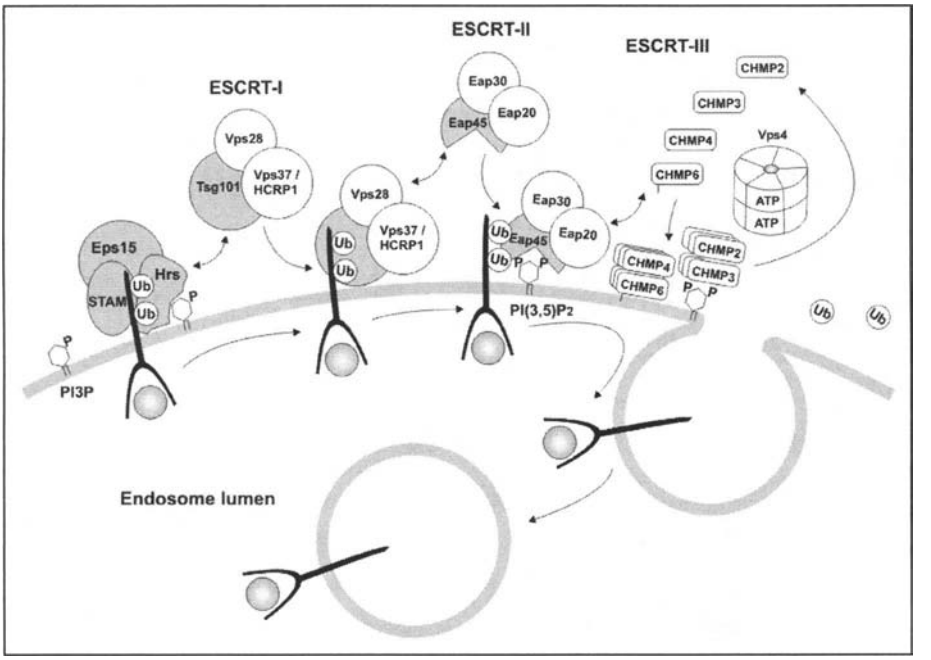

Figure 3. Ubiquitin-mediated sorting into the degradative pathway A ubiquitinated membrane protein in the endosome membrane is recognised by a Hrs-containing complex. The cargo is delivered first to ESCRT-I, thereafter to ESCRT-II and finally transported into intraluminal invaginations via the function of ESCRT-III. Disassembly of ESCRT-III is mediated by the ATPase Vps4.[51,109] The phosphoinositides phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinositol 3,5-bisphosphate [PI(3,5)P_2] participate in the membrane recruitment of Hrs and ESCRTs, respectively.[33,37,110] Ubiquitin-binding proteins are shaded in gray. Double arrows indicate interactions between various complexes. Ub, ubiquitin.

So far, it is not known whether such cargo requires its own sorting machinery, whether it can piggy-back on ubiquitinated cargo, or whether the Hrs/ESCRT-containing sorting machinery can directly recognise even nonubiquitinated cargo. A GPCR, the δ-opioid receptor, is targeted for lysosomal degradation by a mechanism that does not require ubiqutination of the receptor. Interestingly, the lysosomal targeting of this receptor appears to require Hrs but not ESCRT-I.[53] Hrs is thus an extremely versatile sorting receptor since it is used for endocytic recycling of TfRs, β2-adrenergic receptors and μ-opioid receptors, degradative sorting of ubiquitinated cargoes, as well as for degradative sorting of nonubiquinated cargoes (see Table 1). The various sorting functions of Hrs are probably mediated by distinct domains. In addition to Hrs, the ubiquitin-independent endosomal sorting machinery is likely to use some unique components not found in the ubiquitin-dependent cargo-sorting machinery. For example, the GPCR associated sorting protein (GASP) binds to the cytoplasmic tail of the δ-opioid receptor, and this interaction appears necessary for the lysosomal targeting of the receptor.[54] However, so far it is not known whether GASP and Hrs operate in the same sorting pathway of nonubiquitinated cargo.

Sorting from EEs to LEs does not exclusively occur via inclusion into intraluminal vesicles. An alternative pathway, followed by resident lysosomal membrane proteins such as LAMP-1, appears to involve tubular extensions of the EE.[55] Even though such extensions have typically been associated with endocytic recycling, recent evidence suggests that LAMP-1 is enriched in

specific endosomal tubules that contain the adaptor complex AP-3. Moreover, knockdown of AP-3 strongly increases recycling of LAMP-1 at the expense of its transport to lysosomes.[55] This suggests the existence of a specialized AP-3- and tubule-driven sorting pathway for resident lysosomal membrane proteins.

Sorting to the Biosynthetic Pathway

Like trafficking between the plasma membrane and endosomes, trafficking between the biosynthetic and endocytic pathways is bidirectional. The mannose 6-phosphate receptor (M6PR) is a well-studied example of a membrane protein that shuttles between the biosynthetic and endocytic pathways.[56] Upon delivery of newly synthesised lysosomal hydrolases to the acidic lumen of endosomes, the M6PR is recycled from these organelles to the TGN where it can capture new cargo. The sorting of M6PR in LEs is mediated by the tail-interacting protein of 47 kDa (TIP47), which binds to the cytoplasmic tail of the M6PR.[57] This binding is cooperative with a binding of TIP47 to the active (GTP-bound) form of Rab9, which controls vesicle trafficking between LEs and the TGN, suggesting a coupling between the budding of MP6R-containing vesicles and the function of Rab9.[58] According to the current models of endosomal protein sorting, the localisation of M6PR within LE membranes is somewhat surprising. The receptor can be detected on intraluminal invaginations or vesicles,[59] structures that are reminiscent to those involved in sorting to the degradative pathway.[3,42] This indicates that distinct subpopulations of intraluminal invaginations/vesicles exist, and that intraluminal membranes are not necessarily destined for degradation (see chapter 2 for a detailed discussion about the dynamics of intraluminal vesicles). The biogenesis of the carriers for trafficking between LEs and the TGN remains to be clarified.

M6PRs are not only recycled from LEs to the TGN - such transport can also occur from EEs. An evolutionarily conserved sorting complex, retromer,[60] is mainly localised to tubular extensions of EEs in mammalian cells.[61] This complex consists of the PI3P-binding sorting nexins 1 and 2 and the sorting components hVps26, hVps29 and hVps36,[62,63] of which the hVps35 component binds to the cytoplasmic tail of the M6PR. Depletion of retromer by RNA interference increases the lysosomal turnover of the M6PR, decreases cellular levels of lysosomal hydrolases, and causes swelling of lysosomes. These observations indicate that retromer prevents the delivery of the M6PR to lysosomes, probably by sequestration into endosome-derived tubules from where the receptor returns to the TGN.[61,64] The apparent lack of colocalization between retromer and Rab9/TIP47 suggests that these complexes are involved in M6PR retrieval at distinct locations. This raises the possibility that retromer and TIP47/Rab9 could function sequentially in endosome-to-TGN retrieval of M6PR as a mechanism to prevent mis-sorting of M6PRs to lysosomes. It is interesting to note that yeast Vps10, a sorting receptor for carboxypeptidase Y that bears structural and functional similarities to the mammalian M6PR, requires retromer for its trafficking from endosomes to the TGN.[65] Since yeast has no direct counterpart of Rab9, this suggests that the retromer has appeared earlier in evolution than Rab9/TIP47 for endosome to TGN sorting of M6PR-like molecules.

The trafficking of many bacterial and plant toxins that use cellular endocytotic and intracellular sorting machineries to invade cells has shed additional light on the understanding of mechanisms of endosomal sorting. Two toxins that are potent inhibitors of protein synthesis, the plant toxin ricin and the bacterial Shiga toxin, are transported from endosomes to the TGN by Rab9-independent mechanisms.[66] Endosomal tubules frequently contain clathrin-coated buds,[67] and clathrin depletion strongly inhibits trafficking of Shiga toxin from endosomes to the TGN, whereas endocytosis of the toxin is less affected.[68,69] In addition to clathrin, the clathrin-binding protein EpsinR is also required for retrograde trafficking of Shiga toxin, whereas the clathrin adaptor AP-1 is not required.[68] Although Rab9 is not required, other Rab GTPases have been found to regulate the retrograde trafficking of Shiga toxin: Rab11, which regulates trafficking through the RE, is involved in the retrograde trafficking of Shiga toxin.[70] While the small GTPase Rab6 has been reported to control retrograde trafficking within the Golgi

apparatus, a closely related splice variant, Rab6', has been found to regulate the trafficking of Shiga toxin from endosomes to the TGN.[71] Even though the retrograde trafficking of Shiga toxin is clearly clathrin- and Rab11-dependent, this is not the case with ricin.[72] This indicates that, although both these toxins exploit trafficking pathways from endosomes to the TGN, they follow different trafficking routes. Taken together with the data on M6PR trafficking, this indicates that there are multiple trafficking routes from endosomes to the TGN. This points to the existence of several distinct sorting machineries, of which only few subcomponents are known at the moment.

Conclusions and Perspectives

Accumulating evidence suggests that endosomal cargo molecules may be sorted along several alternative pathways (Fig. 1), specified by signals embedded within the cargo structure. These signals are surprisingly diverse, as are the machineries that recognize them (Table 1). The emerging picture is that there are several parallel pathways that mediate endocytic sorting to the recycling, degradative and biosynthtic pathways, and that multiple specific carriers are involved. Some molecules, such as the M6PRs, are sorted along at least two independent pathways out of endosomes, perhaps in order to ensure efficient retrieval. It is going to be a great challenge to untangle the molecular mechanisms that distinguish various sorting pathways.

For simplicity, we have in this review discussed endosomal sorting in nonpolarized cells such as fibroblasts. The situation is even more complicated in polarized cells such as neurons and epithelial cells. Here, specialized endosomal pathways exist, including the sorting of endocytosed proteins into regenerating synaptic vesicles[73] and the communication between apical and basolateral endosomes.[74] We also have not discussed certain less-characterized pathways, like the direct sorting of endocytosed SV40 virus from endosomes/caveosomes to the endoplasmic reticulum,[75] and the trafficking of MHC class II molecules from LEs/lysosomes to the plasma membrane of dendritic cells.[76] This multitude of endosomal trafficking pathways is likely to be mirrored by an increasing complexity of sorting signals and sorting machineries. These machineries may not only include protein complexes, as discussed here, but also membrane lipids that cluster into "rafts".[77]

One of the best-characterized sorting mechanisms in the endosome involves the degradative sorting of mono- or multi-ubiquitinated membrane proteins by a conserved machinery that involves Hrs and ESCRT proteins.[3,42] However, even in this case, we only have an incomplete picture of the sorting mechanisms. Why are some cargoes ubiquitinated and other not? Even though ubiquitin ligases that append ubiquitin to specific endosomal cargoes have been identified,[78-80] it has been surprisingly difficult to identify consensus sequence motifs that are targeted for ubiquitination. Presumably, the signals that specify ubiquitination are encoded by three-dimensional determinants and therefore difficult to identify by sequence analyses alone. Another conceptual problem that concerns the ubiquitin-recognition machinery is the idea that ubiquitinated cargo is delivered from one protein complex to another. How is this process driven in the right direction? It is possible that interactions between the sorting machinery and ubiquitin are regulated by their phosphorylation or ubiquitination,[81] although this remains to be determined. For other sorting steps, such as retrograde trafficking to the biosynthetic pathway, our knowledge is even less complete at the moment. Nevertheless, even if our view of the sorting signals and machineries is still very fragmentary, the ongoing functional genomic analyses and structural determinations of cargo-machinery interactions are likely to yield substantial new information within a short space of time.

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CHAPTER 8

Signaling from Internalized Receptors

Simona Polo, Letizia Lanzetti and Silvia Giordano*

Abstract

Activation of many receptors triggers a cascade of signal transducing events and increases their rate of internalization. Receptor endocytosis has always been viewed primarily as a mechanism to negatively regulate receptor activation, but recent evidence suggests that internalization may result in the formation of specialized signaling platforms on intracellular vesicles. Thus, the investigation of the molecular composition of the various vesicular compartments, their interplay and their spatial and temporal regulation is crucial in order to fully understand the modality of cell signaling.

Introduction

Cells sense and respond to extracellular signals via a dynamic signal-transduction system capable of supporting or inhibiting cell activation. Efficient delivery of signals from the extracellular environment to the intracellular compartment is critical for a tight control of cell growth and differentiation. The first cellular components involved in this signal-transduction system are cell surface receptors, which come in contact with extracellular stimuli, delivered as soluble or membrane bound ligands. It has been known for years that the interaction between receptors and their ligands triggers a cascade of intracellular signals that ultimately leads to cell proliferation. These pathways have been deeply studied and now we have a comprehensive, although not definitive, picture of how they work and of which biological responses are elicited as consequence of their activation. However, only recently it became clear that activated receptors also promote a series of events leading to their endocytosis. Upon internalization, activated receptors are sorted to the endosomes and can be either recycled or degraded in the endosomal or the proteosomal compartments.[1,2] Originally, endocytosis has been considered simply a bowl to eliminate the signaling complexes, but it is now known that internalized receptors are still active and can interact with intracellular transducers and activate new signaling pathways. Since it is currently clear that the output of a transduction process depends not only on the "quality" of the activated signal, but also on its strength and on the location of the emitted signal, new interest on the study of the endocytic trafficking has arisen. The endocytic pathway can achieve signaling compartimentalization since it is organized in a net of distinct but interconnected membrane domains and recent works suggest that it can play a direct role in signal propagation and control.

The rising questions are thus: are the signals originated in the endosomal compartment required for receptors-induced biological responses? Are these signals different from those originated in the plasma membrane? Is there any difference, in term of signal transduction, if the

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receptors are internalized through different pathways (i.e., coated vesicles vs caveolae)? The answers to all these questions are not yet clear, but we are starting to understand that receptor internalization is not simply a way to remove activated receptors from the plasma membrane and that the internalization pathway followed by the receptor can influence the signaling ability of the receptor itself, ultimately leading to different biological responses.[3-6] Moreover, these processes differ from receptor to receptor and the common machinery can be utilized by the different receptors to obtain different outcomes.

In this chapter we will thus describe separately some of the receptors for which the role of internalization on signaling ability is better known.

RTKS: Temporal and Spatial Regulation of Signaling

Receptors for most growth factors are transmembrane proteins endowed with tyrosine kinase activity (receptor tyrosine kinases, RTKs). On ligand binding, RTKs undergo dimerization that results in promotion of their enzymatic activity. RTK-mediated phosphorylation of tyrosine residues on the receptor itself or on intracellular proteins creates binding sites for other proteins containing phosphotyrosine-binding motives.[7] These intracellular substrates can be either enzymes or adaptors or transcriptional factors. The change of enzymatic activity and the modification of membrane lipids occurring as a result of receptor activation originate a network of protein-protein and protein-lipid interactions, phosphorylations and dephosphorylations, modification of cellular compartmentalization, which ultimately leads to changes in gene transcription and to activation of cell proliferation and/or differentiation. As previously mentioned, upon ligand binding and activation, RTKs undergo endocytosis and move through a series of endocytic compartments.

The possibility that endocytic membrane transport has a role in cell signaling has been extensively studied and several experimental evidences now point to this conclusion (reviewed in ref. 4,5,8). One obvious role for endocytosis in signaling could be to provide a spatial regulation of the signaling. One the best example of this function derived from the study of the TrkA system, the receptor for nerve growth factor (NGF), a neurotrophin that functions as a neuronal survival and differentiation factor (Fig. 1A). When NGF is applied selectively to the tip of the axon, the signal has to travel to reach the cell body in order to modify gene transcription; this cannot be achieved by simple diffusion of the signal, but requires microtubule-mediated retrograde transport to the neural cell body of both TrkA and the signaling molecules.[9] Accumulation in the cell bodies of retrogradely transported TrkA and NGF is thus required for neuronal survival while NGF stimulation of the cell body is not sufficient to induce this biological response.[10] Moreover, while NGF and NT-3 (Neurotrophin 3) exert their effect through the same receptor – TrkA - they control unique aspects of neuronal development through differential TrkA internalization and retrograde signaling. NT-3 signals via cell surface TrkA to support axon growth but not survival, whereas NGF, produced by the neuronal final target after synaptic development, supports not only local axon growth, but also survival, anabolic responses and gene expression through retrograde signaling. It has been shown that NGF treatment leads to endocytosis and retrograde accumulation of activated TrkA, Erk1/2 and Akt complexes, whereas NT-3 fails to induce formation of signaling endosomes, but stimulates vesicle trafficking locally within the axon terminal.[11] This differential control of TrkA trafficking ensures that target-derived NGF and not intermediate target-derived NT-3 is solely responsible for retrograde survival signaling.

Always dealing with "spatial regulation of signaling", it has to be considered that RTK signaling is accomplished by several layers of protein-protein interactions. When the RTK becomes activated and undergoes internalization, the full signaling complex is removed from the cell membrane. The result of this event is that only some of the receptor-bound molecules can find their substrates also in the endosomal compartment and thus can continue to signal. For example, the accumulation of the Grb2-SOS complex in endosomes might serve to sustain for a prolonged time the activity of Ras, which can be either constitutively associated with

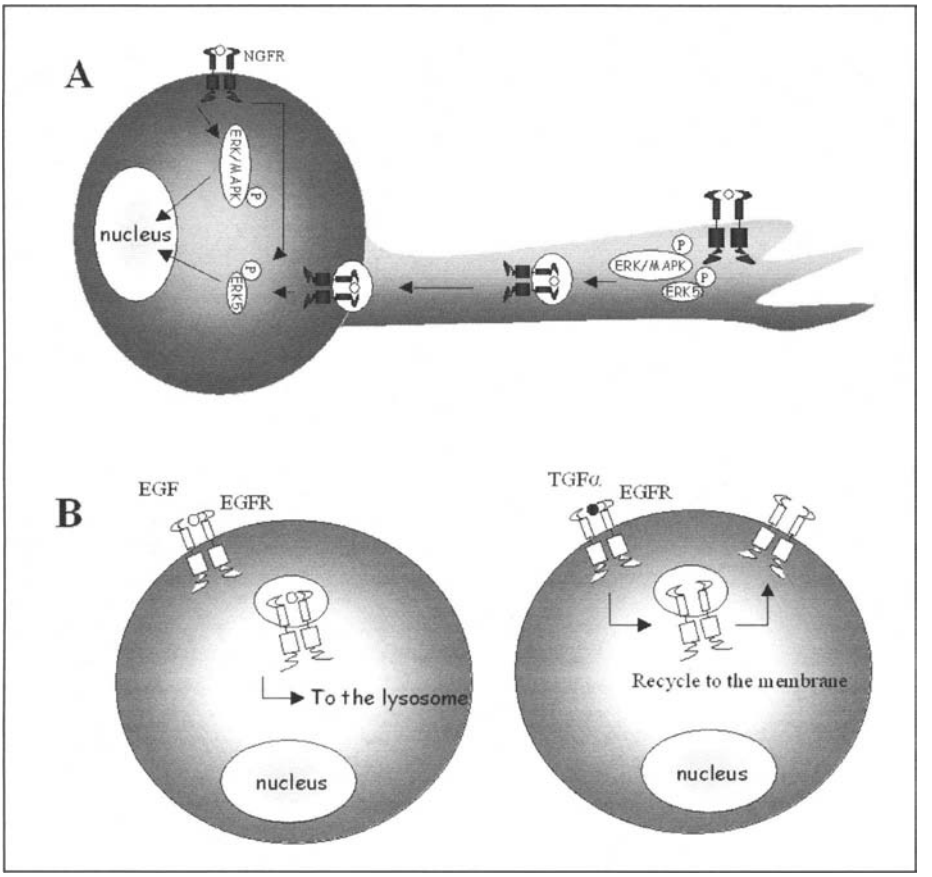

Figure 1. Spatial and temporal regulation of RT signaling. The TrkA system represents one of the best examples of spatial regulation of the signal. When NGF is applied selectively to the tip of the axon, the signal has to travel to reach the cell body in order to modify gene transcription; this cannot be achieved by simple diffusion of the signal but requires microtubule-mediated retrograde transport to the neural cell body of both TrkA and the signaling molecules (modified from: Signal transduction and endocytosis: close encounters of many kinds, Alexander Sorkin and Mark von Zastrow; Nature Reviews Molecular Cell Biology 3, 600-614) B. Endosomes regulate the duration of the signal, controlling the interaction between ligand-receptor pairs. EGFR can interact with several ligands, endowed with different biological abilities. The interaction between EGFR and EGF is quite stable and keeps EGFR in an active state that leads to lysosomal degradation. The instability of the interaction between TGFα and EGFR in the mildly acidic early endosomes does not support receptor sorting into internal multivesicular bodies compartment but rather promotes recycling which results in an increased amount of receptor available on the cell membrane.

endosomes or internalized in response to stimulation. Several effectors of Ras, such as Raf1 and Rab5, are indeed present in the endosomal compartment and can thus be affected by Ras activation.[12-14] On the contrary, when RTK-activated enzymes such as Phospholipase Cγ1 (PLCγ1) and phosphatidyl-inositol 3 kinase (PI3K) are located on the endosomes they are spatially separated from their substrates, which are mainly located at the plasma membrane, and are thus inactive.[15,16] In this way, moving of the RTK signaling complexes to the endosomal

compartment can preferentially allow the maintenance of some pathway while inactivating other ones. Another way to differentially assemble protein complexes involves the action of compartment-specific adaptor proteins, capable of linking activated receptors with distinct sets of accessory and effector proteins. As an example, a late endosomal protein, p14, has been shown to be indispensable for epidermal growth factor receptor (EGFR)-mediated efficient ERK activation. In fact, p14 anchors MP1, a MAPK-scaffold protein, to the late endosome,[17] where MP1 specifically binds to MEK1 and thus facilitates activation of Erks. Interference with p14 expression causes displacement of MP1 to the cytosol and prevents full activation of the MEK-Erk cascade, without impairing early Erk1/2 activation occurring at the plasma membrane.

A definitive proof that endosomal signaling of epithelial growth factor receptor stimulates signal transduction pathways leading to biological responses came from the study of Wang and colleagues.[18] By using an experimental system able to dissect signaling originating from the plasma membrane or from the endosomes they showed that the signal transduced from internalized EGFR, with or without a contribution from the plasma membrane, fully satisfy the physiological requirements for S-phase entry.

Another interesting function of endocytosis in signal tranduction is to temporally regulate the length of the signal since its duration is an important parameter to determine the biological outcome. The balance between the number of receptors that undergo degradation versus those that are recycled to the cell membrane is critical to determine the strength of the signal. To this matter, important differences have been observed also among receptors belonging to the same family. The best-studied example is, by far, that of the EGFR family, formed by four structurally related receptors[19] (Fig. 2B). The most oncogenic member of this family, HER2, is poorly downregulated upon activation and recycles very efficiently. On the contrary, EGFR homodimers are effectively directed to a degradative fate following ligand binding, but when EGFR is heterodimerized with HER2 it preferentially undertakes a recycling fate. This event changes the duration of the signaling since new receptors are always exposed on the membrane where they are available for the ligand. Moreover, EGFR signals from endosomes for most of its lifetime, while the other members of the family remain active for longer periods on the plasma membrane.

Endosomes regulate the duration of the signal also controlling the interaction between the ligand-receptor pairs. For example, EGFR can interact with several ligands, endowed with different biological abilities. The stronger mitogenic activity of TGFα (Transforming Growth Factor α) versus EGF is explained on the basis of their differential sensitivity to acidic endosomal pH, that affects the stability of the interaction with EGFR and, thus, its intracellular trafficking.[20] In fact, the instability of the interaction between TGFα and EGFR in the mildly acidic early endosomes does not support receptor sorting into internal multivesicular bodies compartment, but rather promotes recycling which results in an increased amount of receptor available on the cell membrane.

Notch: Endocytosis is Required for Signaling

The family of the Notch receptors represents the prototype of cell surface receptors that require endocytosis in order to signal. Notch signaling plays a fundamental role in regulating cell-fate specification in a variety of developmental and homeostatic processes.[21,22] Alterations in Notch signaling lead to unbalance of these processes and have been implicated in tumorigenesis.[23]

Notch receptors (Notch1-Notch4 in vertebrates) are single-pass membrane receptors that are activated by the Delta/Serrate/Lag2 families of transmembrane ligands, located on the surface of a neighbor cell. The engagement of the extracellular portion of Notch in binding to Delta induces two subsequent proteolytic cleavages[21] (Fig. 2). The first cleavage occurs in the extracellular domain of Notch and it depends on the activity of the TNFα-converting enzyme (TACE).[24,25] The result is the transendocytosis of the Notch extracellular domain/Delta

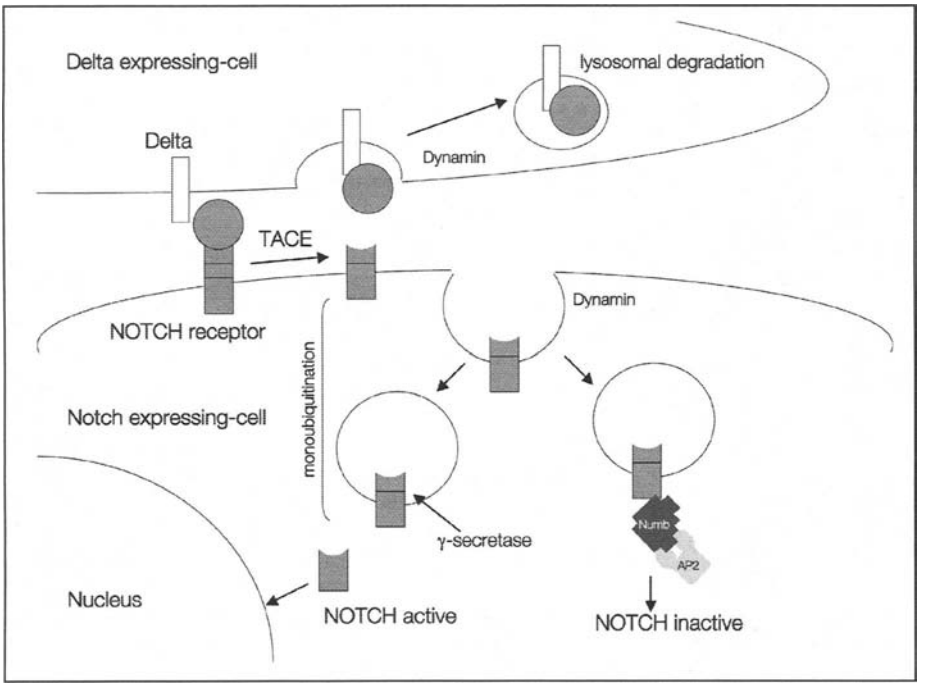

Figure 2. Endocytosis regulates Notch receptor signaling. Notch binds through its extracellular domain to Delta that is expressed on the surface of the signal-sending cell. The binding results in the first cleavage operated by the TACE enzyme (TNFα-converting enzyme) in the extracellular domain of Notch. The Notch extracellular domain is internalized, together with Delta, in the Delta-expressing cell, in a dynamin-dependent manner. The remaining membrane-tethered Notch is cleaved by γ-secretase depending on both monoubiquitination and endocytosis. It causes the release of the intracytoplasmic domain of Notch that translocates into the nucleus where it regulates transcription. Alternatively endocytosis may downregulate Notch activity by removing the receptor from the cell surface via the interaction with the endocytic protein Numb. (modified from Polo S, Pece S, Di Fiore PP. Endocytosis and cancer. Curr Opin Cell Biol. 2004, 2:156-61).

complex into the Delta-expressing cell. This initial endocytic event is a prerequisite for Notch activation in the Notch-expressing cell, since Delta mutants that cannot be internalized, are unable to activate Notch "in vivo".[26] The second cleavage occurs in the transmembrane domain of the remaining membrane-tethered Notch and it is operated by the presenilin/γ-secretase complex. It causes the release of the intracytoplasmatic domain of Notch that translocates into the nucleus where it regulates transcription[27,28] (Fig. 2). Indeed it has been demonstrated that inhibition of endocytosis, by means of dominant-negative mutants such as Dynamin II K44A or Eps15DN, prevents the translocation of the Notch intracytoplasmatic domain into the nucleus.[29,30] Therefore endocytosis is required for proper Notch signaling both in the ligand-expressing cell as well as in the signal-receiving cell.[29]

In addition, Notch is post-translationally modified upon appendage of an ubiquitin moiety to a lysine residue in the juxtamembrane region.[30] Presenilins are able to interact with the monoubiquitinated form of Notch and this step is required for the γ-secretase cleavage.[30] As a pool of presenilins has been found at the cell surface and in the endocytic compartments[31,32] it

is conceivable that monoubiquitination and endocytosis of the receptor are necessary for driving Notch to compartments where γ-secretase cleavage can operate.[30]

Endocytosis is therefore acting as a "positive" mechanism in promoting the delivery of the Notch signal to the cell. Nevertheless, Notch signaling is also controlled by endocytosis through a more "classical" mechanism, namely receptor downregulation. A critical player in influencing the Notch availability at the plasma membrane is represented by Numb, an endocytic protein that binds to α-adaptin and localizes to the endosomes in mammalian cells.[33] Numb physically interacts and antagonizes Notch[33,34] as demonstrated in the sensor organ precursor cell of *Drosophila,* where asymmetric partition of Numb and α adaptin at mitosis results in Notch silencing in the Numb-receiving cell and, consequently, in the acquisition of different cell fate.[35] The Numb/Notch antagonism is relevant also in mammals, in particular in the control of tumor proliferation. Increased Notch signaling is observed in Numb-negative tumors, where it can be reverted to basal levels after enforced expression of Numb.[36]

In conclusion, endocytosis regulates Notch signaling at multiple levels: i) modulating Notch signaling via the transendocytosis of the Notch extracellular domain/Delta complex into the ligand presenting cell; ii) controlling the γ-secretase cleavage and therefore the release of the Notch intracellular domain; iii) down-regulating Notch signaling by affecting receptor availability at the plasma membrane.

Different Endocytic Route Different Signaling: The TGF-βR Paradigm

Until recently, it was widely accepted that all cell-surface receptors follow the same endocytic pathway and that they are internalized by clathrin-coated pits, with sorting at the cell surface being achieved solely through the direct or indirect binding of receptor cytoplasmic domains to clathrin associated proteins. However, clathrin-independent ways of entry into the cell also exist. In particular, progress has been made in characterizing a pathway involving cholesterol- and glycosphingolipid-rich membrane domains (rafts) and in identifying their cargoes.[37] It was shown that the TGF-β (Transforming Growth Factor β) internalizes through both coated pits and caveolae. The former route is associated with increased receptor signaling from early endosomes, while the caveolar pathway causes rapid receptor degradation.[38] Thus, depending on the entry route, the fate of internalized TGF-β receptors will be different (Fig. 3).

At the molecular level activated TGF-β receptors bind and phosphorylate R-Smads (Smad2 and Smad3). R-Smad binding to the receptors is facilitated by a protein called SARA (Smad Anchor for Receptor Activation) which has a FYVE domain and is predominantly localized to the PitdIns3P-enriched endosomes, a key compartment for Smad activation.[39] Clathrin-dependent internalization of the TGF-β receptor into EEA1-positive early endosomes enables the engagement of TGF-β receptor by SARA, necessary to phosphorylate Smad2 and to achieve subsequent propagation of the signal (Fig. 3A). Indeed, interfering with clathrin-dependent trafficking, using K^+ depletion, or dominant negative mutants of dynamin or eps15 blocks TGF-β-induced Smad2 activation and nuclear translocation.[38]

On the other end, the other pool of the receptor is internalized via clathrin-independent route, namely a lipid raft–caveolar internalization pathway, which involves caveolin-1-positive vesicles called caveosome. In caveosomes, TGF-β receptors do not encounter SARA, but the ubiquitin ligase Smad7–Smurf2 complex that colocalizes with caveolin-1 and preferentially associates with receptors in rafts.[38] Indeed, TGF-β receptor turnover is inhibited by lipid rafts disruption and this strongly indicates that this pathway leads to degradation and counteracts the clathrin–mediated signaling (Fig. 3B).

Therefore, it seems that Smad signaling components are segregated into two internalization pathways, where they differentially regulate TGF-β signaling. Early endosomes represent a signaling center that functions both to sequester active receptor complexes from rafts and caveolin and to promote access to the Smad2 substrate via SARA, whereas the raft–caveolin compartment

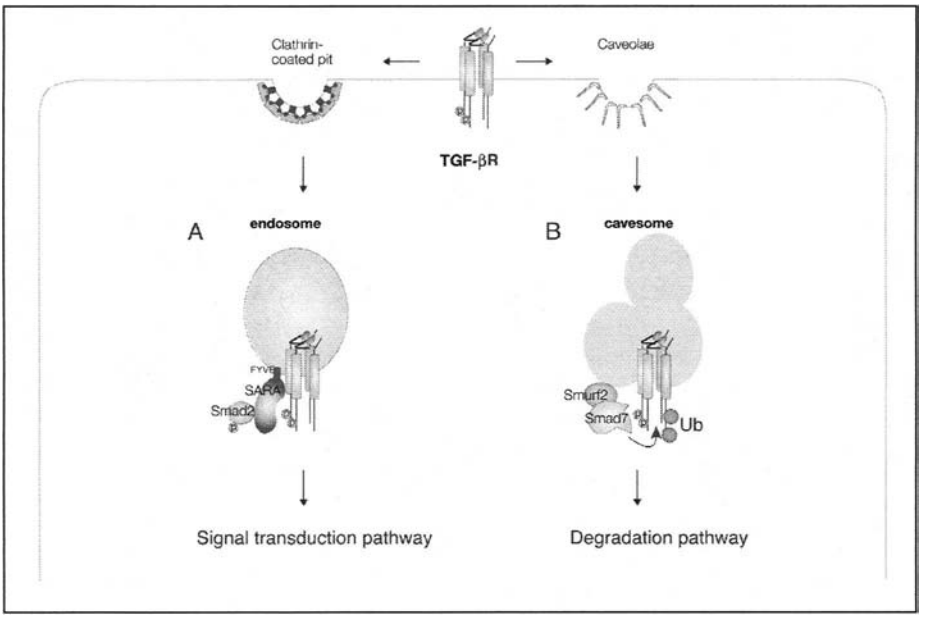

Figure 3. Alternative entry routes for TGF-β receptor. A In the clathrin-mediated pathway, receptor is directed towards the early endosomes, where it interact with Smad2 and SARA. From these vesicles, TGF-βR is able to signal and is recycled back to the cell surface. B In the caveolar-mediated pathway, receptor is directed towards the caveosomes, where it encounter the ubiquitin ligase Smad7–Smurf2 complex and become ubiquitinated and degraded.

may represent a previously undescribed compartment that regulates ubiquitin-dependent degradation of membrane receptors.

An intriguing observation is that expression of constitutively inactive Rab5 (Rab5S34N) stimulates TGF-β signaling, while expression of constitutively active Rab5 (Rab5Q79L) has no effect.[40] One possibility is that Rab5 inhibition may modify the transport of TGF-β receptors between caveosomes and early endosomes, thus underscoring the influence of signaling on trafficking.

Recently, it was also shown that the EGF receptor internalizes through both coated pits and caveolae depending on the level of activation (Sigismund et al, PNAS in press). When the receptor is stimulated with low doses of EGF, is internalized almost exclusively through the clathrin pathway, and it is not ubiquitinated. At higher concentrations of ligand, however, a substantial fraction of the receptor is endocytosed through a lipid raft/caveolar-dependent route, as the receptor becomes ubiquitinated. Interestingly, at low levels of EGF, the EGFR is already fully competent of signaling via its effectors, whereas at high levels of EGF (when clathrin-independent endocytosis becomes significant) there is no increase in signaling abilities, but readily detectable increase in EGFR downregulation. Thus, the combined analysis of TGF-βR and EGFR data suggest the intriguing possibility that the caveolar/raft internalization does not contribute to signaling, and it is preferentially associated with receptor degradation, via ubiquitination.

GPCR and β-Arrestin: Signaling from the Endosomes

Signaling mediated by GPCRs (G protein-coupled receptors) begins with the activation of the receptor through the binding of agonist; this leads to a conformational change within the receptor intracellular domains, which can then be recognized by intracellular proteins.[41] The

most common GPCR signal transducing proteins are the heterotrimeric G proteins, which in turn can activate a wide spectrum of effector molecules, including phosphodiesterases, phospholipases, adenylyl cyclases and ion channels.[42] Active GPCRs are also the target of G protein-coupled receptor kinases, which phosphorylate the receptors leading to the rapid recruitment and binding of cytosolic arrestins (known as β-arrestin-1 and -2).[43]

β-arrestins are key players for receptor desensitization and internalization since β-arrestin binding to GPCRs both uncouples receptors from heterotrimeric G proteins and targets them to clathrin-coated pits for endocytosis[44-46] (Fig. 4A). In addition to this established role, the isolation from intact cells of protein complexes containing specific GPCRs, β-arrestins and either Src or ERK/MAPKs has led to the hypothesis that endosome-associated β-arrestins function as molecular scaffolds for the assembly of specific kinase cascades and, perhaps, for the recruitment of other signaling molecules[47,48] that mediate GPCR signaling from endosomes (Fig. 4B). This complex, in fact, has been proposed to mediate a distinct "second wave" of signal transduction through MAPKs, which occurs after the "classical" pathways (such as signaling via adenylyl cyclase) have already been originated at the cell surface.[49] Proteinase-activated receptor 2 (PAR2),[48] angiotensin AT1A receptor (AT1AR)[47] and neurokinin receptor 1 (NK1R)[50] are examples of GPCR for which scaffolding interactions that involve β-arrestins in the ensosomes have been described (Fig. 4B).

Interestingly, activation of MAPK by the heptahelical μ opioid receptor does not require endocytosis of the heptahelical receptor,[51] yet MAPK activation by this receptor is strongly inhibited by a dominant-negative mutant dynamin that blocks endocytosis via coated pits.[51,52] This indicates that dynamin-dependent endocytosis of another molecule might be required for ERK/MAPK signaling by GPCRs, or that dynamin might mediate another function in signaling that is independent of endocytosis per se. Indeed, in some cases, ERK/MAPK signaling by GPCRs is mediated by transactivation of an RTK such as the EGF receptor,[53] and endocytosis of the RTK — but not the GPCR — could be the crucial event that is involved in signal transduction.

It has also been observed that overexpression of mutant dynamin does not detectably affect receptor-mediated activation of Ras and Raf, whereas phosphorylation of ERK/MAPK 1/2 by MEK is strongly inhibited.[54] Therefore, it was postulated that the endosomal localization of MEK, perhaps by endocytic transport of a putative MEK membrane anchor, is essential for ERK/MAPK 1/2 activation. These considerations have led to a search for more specific ways of manipulating endocytic transport of a specific receptor or signaling molecule. One approach has been to examine the effects of mutant βarrestins, which specifically inhibit GPCRs endocytosis without affecting endocytosis of other molecules such as RTKs.[55] However βarrestins, like mutant dynamin, can engage numerous interactions, including those with scaffold signaling kinases,[47,56] so this originates some ambiguities in functional studies of GPCR signaling performed with the use of the inhibitors currently available.

Conclusions

A growing body of evidence points out the role of key signaling molecules in modulating multiple and different signal transduction pathways. The capability to transduce distinct signals relies mainly on the spatial and/or temporal regulation of the molecules. The plasma membrane has long been considered the major signaling-emanating compartment due to the presence of the transmembrane receptors that are engaged by extracellular ligands and in turn initiate the signaling cascade by recruiting downstream mediators and by activating GTPases and other enzymes. Nevertheless, it has been demonstrated that signaling platforms are also assembled on early endosomes and that they can propagate signals inside the cell. In addition other intracellular membrane compartments appear to be responsible for signal propagation like for instance the Golgi apparatus. The Golgi is involved in protein maturation and secretion, thus controlling the inside-out signaling. The discovery that the GTPase Ras (Ha-Ras) localizes at the Golgi and signals from this compartment reveals a role for the Golgi also in the

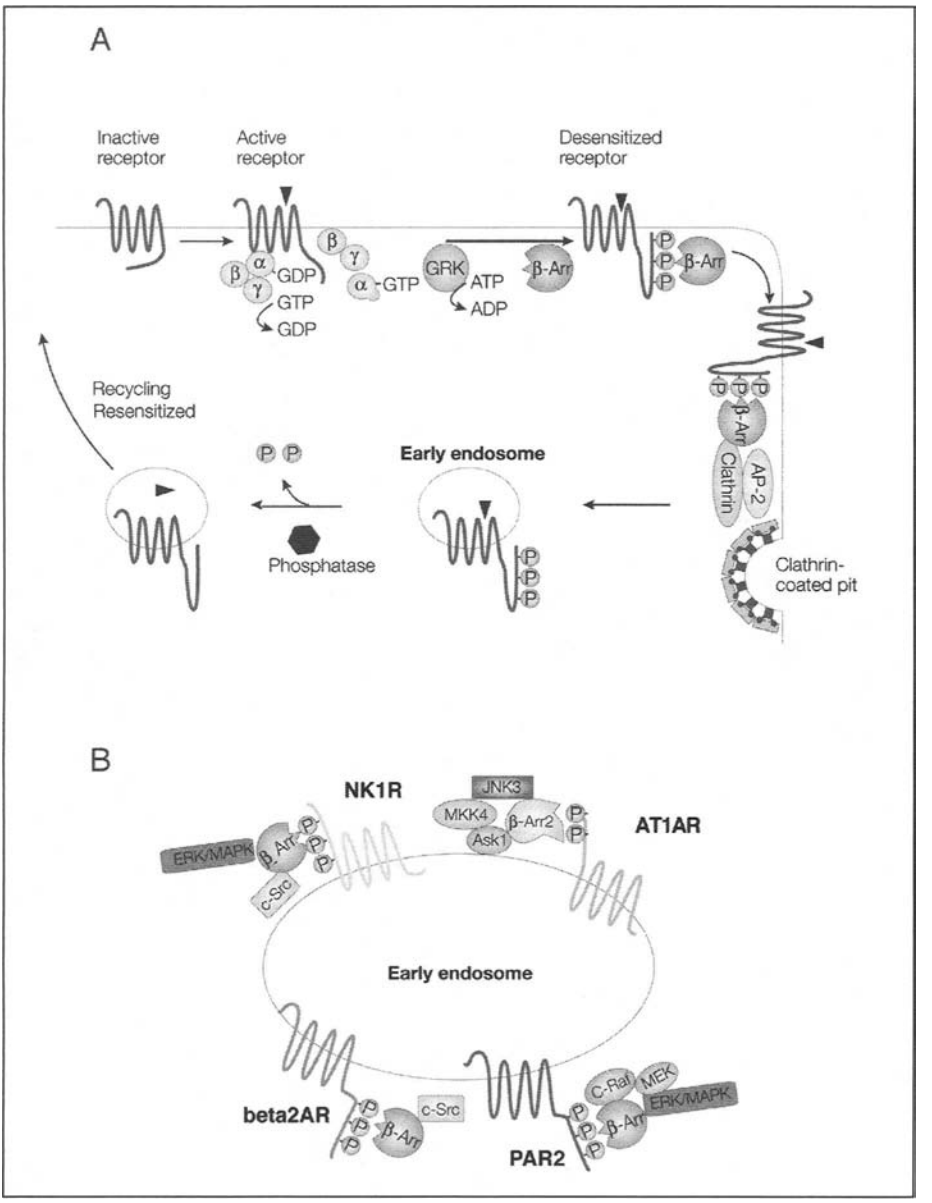

Figure 4. GPCR activity at the membrane and in the endosomes. A) At the membrane activated GPCR interact with the heterotrimeric G proteins, which in turn, activate the signaling cascade. Upon activation GPCR are desensitized by phosphorylation and interact with β-arrestins. β-arrestins promote receptor internalization via clathrin-mediated pathway into endosomes where receptors are dephosphorylated and recycled back to the membrane (resensitization). B) In the endosomes GPCR, through β-arrestins, can interact with various component of the signaling cascade, such as Src and ERK/MAPK kinases, leading to a "second wave" of signal transduction. (modified from: Signal transduction and endocytosis: close encounters of many kinds, Alexander Sorkin and Mark von Zastrow; Nature Reviews Molecular Cell Biology 3, 600-614).

outside-in signaling.[57,58] Therefore the localization or the relocalization of signaling molecules to vesicular compartments is a way to increase signal complexity.

Although the spatial regulation of signaling molecules cannot be, probably, distinguished from a "temporal" regulation, compartmentalization remains an important mechanism of controlling signaling. The temporal regulation of vesicles-derived signals represents an active field of investigation in particular concerning the requirement for vesicular trafficking during the process of cell division (reviewed in ref. 59).

In conclusion the new findings discussed in this chapter argue for a complex, bidirectional cross-talk between signaling and membrane-transport network. Further investigation of the molecular composition of the various vesicular compartments, their interplay and their spatial and temporal regulation is needed in order to clarify the modality of cell signaling.

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CHAPTER 9

Endocytosis of Receptor Tyrosine Kinases: Implications for Signal Transduction by Growth Factors

Gal Gur, Yaara Zwang and Yosef Yarden*

Abstract

Growth factors and their respective receptor tyrosine kinases (RTKs) play pivotal roles in normal cellular functions, such as proliferation and motility, as well as in pathogenesis, including cancer. The amplitude and kinetics of growth factor signaling are determined mainly by a highly regulated endocytic process, which sorts activated receptors to degradation in lysosomes. Molecular mechanisms underlying receptor down-regulation are being unraveled: the active receptor recruits Cbl ubiquitin ligases that decorate it with multiple monomers of ubiquitin. In parallel, Nedd4/AIP4 ubiquitin ligases attach ubiquitin to a set of ubiquitin-binding adaptors (e.g., Epsin and the EGF-receptor protein substrate, Eps15) necessary for the assembly of a clathrin coat. Analogous but distinct ubiquitin-binding platforms underlie receptor sorting into shuttling vesicles at the plasma membrane, early endosomes and a prelysosomal compartment called the multi-vesicular body. In addition to ubiquitylation, phopshorylation of both RTKs and coat adaptors orchestrate receptor sorting in concert with machineries responsible for membrane bending and vesicle fusion. The default route diverts internalized receptors back to the plasma membrane, thus enabling prolonged signaling associated with pathological processes. This review concentrates on the epidermal growth factor receptor (EGFR) as a prototype and highlights the major events occurring on its journey to the lysosome.

Introduction

Cell fate determination in embryogenesis, as well as morphogenic processes throughout adulthood, are regulated primarily by polypeptide growth factors. The initial event underlying stimulation of a target cell by growth factors involves their binding to transmembrane receptor tyrosine kinases (RTKs), whose intracellular portions share a catalytic kinase activity specific for tyrosine residues (reviewed in refs. 1-3). Upon binding to the respective growth factor molecule, RTKs undergo dimerization and catalytic stimulation. This enables them to recruit and/or phosphorylate multiple protein substrates, many of which utilize intrinsic phosphotyrosine-binding regions [e.g., Src-homolgy 2 domain (SH2) and phosphotyrosine-binding domain (PTB)] to bind with phosphorylated tyrosine residues of the active receptor.[4] These receptor-centered events instigate a large number of simultaneous biochemical cascades, which collectively transmit extracellular signals to target organelles and culminate in gross cellular alterations (e.g., cell division or migration). Concomitant with signal distribution and propagation, a variety of desensitization processes are launched, such that the balance between positively-acting and

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negatively-acting cascades determines the amplitude and duration of the ensuing biochemical signals (reviewed in ref. 5). Perturbations of this delicate balance often lead to pathogenesis, such as skeletal disorders, cancer and diabetes. We concentrate below on the epidermal growth factor receptor (EGFR) as a prototypic RTK. After briefly describing positively-acting pathways, this chapter will concentrate on the major negatively-acting regulatory process of RTKs, namely: growth factor-induced internalization of active receptors and their subsequent sorting to intracellular degradation.[6-8]

Positively-Acting Signaling Pathways

The most characterized signaling pathways induced upon activation of RTKs, including members of EGFR family (ErbB/HER), are the Ras-mitogen-activated protein kinase (Ras-MAPK), the phosphatidylinositol 3' kinase-protein kinase B (PI3K-PKB/Akt), and the phospholipase C-protein kinase C (PLC-PKC) pathways. The four ErbB proteins, and in fact most RTKs, couple to activation of the Ras-MAPK pathway through SH2 domain-mediated recruitment of the Grb2 adaptor,[9] or indirectly through PTB domain-mediated binding of the Shc adaptor.[10] Regardless of the exact route, active MAPK/Erk molecules translocate to the nucleus to phosphorylate specific transcription factors, such as Sp 1, E2F, Elk-1 and AP1.[11] In a similar manner, receptor phosphorylation provides acceptor sites for the SH2 domain of the regulatory subunit of PI3K, p85. For example, binding of p85 to tyrosine phosphorylated ErbB proteins (predominantly ErbB-3) results in activation of p110, the catalytic subunit of PI3K.[12] Akt (also called protein kinase B, PKB) is a key effector of PI3K; it is recruited to the plasma membrane through its PH domain and activated upon phosphorylation by serine/threonine kinases. The importance of the proliferation and cell survival signals, which are mediated by PI3K, is reflected by the tumor suppressive effects of PTEN. This lipid phosphatase dephosphorylates phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)-P_3], a lipid required for PKB/Akt activation, and undergoes frequent mutational inactivation in human cancer, which results in constitutive activation of Akt.[13] The third cascade leads to activation of protein kinase C (PKC). Phospholipase Cγ (PLCγ) is recruited to the membrane through SH2 domain-mediated binding to activated RTKs, including EGFR and ErbB-2, as well as through binding of its PH domain to PI3K products (reviewed by ref. 14). Subsequent to phosphorylation by RTKs, PLCγ hydrolyzes PI(4,5)P_2 to generate inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (DAG), which are implicated in the mobilization of intracellular calcium ions and activation of PKC, respectively. Several additional signaling pathways are induced by RTKs, including cytoplasmic tyrosine kinases of the Src family and recruitment of transcription factors belonging to the STAT family. Subsequent to their phopshorylation, STAT proteins translocate to the nucleus to activate gene transcription critical for cell proliferation and angiogenesis.

Negatively-Acting Signaling Pathways

Much of the information relevant to attenuation of RTK signaling emerged from studies of invertebrate systems. A single EGFR orthologue and a single EGF-like ligand are found in the worm *C. elegans*, and their signaling is attenuated by a small group of proteins: a Clathrin adaptor, Sli-1 (a c-Cbl orthologue), a GTPase-activating protein called GAP-1 and a cytoplasmic tyrosine kinase homologous to mammalian Ack-1.[15] The single ErbB orthologue of flies is attenuated by similar mechanisms, along with several additional pathways such as an inhibitory ligand, Argos, a family of transmembrane molecules called Kekkons and an adaptor molecule, Sprouty, which has four orthologues in mammals (reviewed by ref. 16). Mammalian RTK signaling is intercepted at multiple additional junctures, which include inhibition of kinase activity by RALT/Mig-6,[17,18] a soluble receptor variant that intercepts receptor activation,[19] a transmembrane inhibitory protein,[20] and specific protein tyrosine phosphatases. Most remarkable is a large family of dual specificity phosphatases called MKPs, which dephosphorylate

specific MAP-kinases.[21] It is notable that expression of both MKPs and RALT/Mig-6, along with additional negative regulators, is rapidly elevated upon RTK activation within the framework of transcription-based negative feedback loops.

Signal Attenuation by Ligand-Induced Endocytosis of RTKs

Concomitant with receptor activation, ligand binding initiates a multi-step process that culminates in receptor degradation. RTKs such as EGFR and ErbB-2 are enriched in membrane microdomains called caveolae. This subset of lipid rafts contains Caveolin proteins, glycosphingolipids, and cholesterol, as well as multiple signaling molecules, including Src family kinases and H-Ras (reviewed in ref. 22). A conserved Caveolin-binding motif within the kinase domain of EGFR mediates the interaction of EGFR with the cytosolic Caveolin scaffolding domain of Caveolin -1 and -3.[23] Further, this interaction may inhibit kinase activity, but ligand binding to ErbB-1 induces migration of active receptors out of caveolae in a process requiring Src family kinases.[24] Subsequently, active RTK molecules aggregate over Clathrin-coated regions of the plasma membrane, where they start their journey to the lysosome, which will be detailed below. It is notable that RTK molecules whose internalization and degradation are defective due to large deletions acquire enhanced mitogenic and oncogenic activities.[25] This and similar observations are consistent with the notion that endocytosis serves to attenuate growth factor signals.

Common Molecular Mechanisms in Receptor Endocytosis: Curvature Sensing and Post-Translational Protein Modifications

Bending of the planar lipid bilayer and two post-translational protein modifications, namely phosphorylation and ubiquitylation, are involved in receptor endocytosis. These machineries, in combination with vesicle budding and fusion, recur along the endocytic itinerary, although distinct sets of protein platforms execute receptor sorting at different steps of endocytosis.

Membrane Bending

The generation of high-curvature lipid-bound transport carriers represented by tubules and vesicles requires physical perturbation of the lipid bilayer, as well as direct interactions between cytosolic proteins and lipid bilayers. Proteinaceous coats selectively associated with the surface of membrane buds are key mediators of vesicle formation in the endocytic pathways: Clathrin oligomerization into a coat scaffold on the membrane forms a polyhedral lattice. Nevertheless, the current notion is that Clathrin can at best serve to maintain an already curved membrane, thereby preventing its collapse back into a planar form.[26] Thus, in addition to coat-protein lattice formation mechanisms that help deforming the bilayer are likely to come into play. The GTPase called Dynamin was found to deform lipid bilayers into narrow tubules coated by Dynamin spirals.[27] Cytosolic proteins like Amphiphysin and Endophilin, two major interactors of Dynamin, were found to deform liposomes in vitro into narrow membrane tubules.[28,29] Both adaptors are able to sense and alter membrane curvature by modifying the lipid content, or by deforming the membrane mechanically via their BAR domains.[30] The BAR domain has a coiled-coil structure that binds preferentially to negatively charged membranes.[31] Epsin, an interactor of Clathrin and of the Clathrin adaptor AP-2, was also shown to induce membrane tubulation.[32] In addition, lipid components of the membrane, either directly or via interaction with proteins, have been suggested to facilitate the structural changes necessary to deform membranes. For example, selective transfer of lipids between bilayer leaflets has been proposed as a mechanism by which surface area asymmetries could influence budding and endocytosis. In addition, certain lipid species are postulated to favor bilayer curvature owing to their intrinsic properties, their relative geometries, or both.[33] Cholesterol, for example, selectively accumulates along the membrane, which may decrease membrane rigidity, create bilayer surface-area discrepancy, and facilitate budding.[34]

Protein Phosphorylation

Protein phosphorylation is a major regulatory mechanism of Clathrin-dependent endocytosis. Serine and threonine phosphorylation of Clathrin coat proteins plays an important role in the organization of macromolecular complexes during Clathrin-mediated endocytosis. Src-dependent tyrosine phosphorylation of coat proteins, such as Clathrin heavy chain and Dynamin, has been observed in cells stimulated with growth factors or hormones, although the precise role for these modifications is not fully understood.[35,36] Likewise, although Eps15 undergoes tyrosine phosphorylation, the precise role of this phosphorylation remains unclear. It appears, however, that phosphorylation-impaired mutants of Eps15 do not interfere with EGFR recruitment to pits, but rather with subsequent phases of the internalization process.[37] Phosphorylation of the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs; renamed Hgs) in response to EGF stimulation takes place on the evolutionary conserved tyrosines 329 and 334. Whereas, the ubiquitin-interacting motif (UIM; see below) of Hgs is required for phosphorylation, this modification is not required for UIM-dependent ubiquitylation.[38]

Protein Ubiquitylation

Modification of plasma membrane proteins by mono-ubiquitylation appears to serve as a signal sufficient to induce internalization and endocytic trafficking.[7,39] In line with this notion, two recent reports demonstrated that in-frame fusion of ubiquitin to EGFR resulted in constitutive internalization and enhanced degradation of the chimeric proteins.[40,41] Further, Cbl was shown to mediate mono-ubiquitylation of EGFR at multiple lysine residues, some localized within the kinase domain. These data demonstrate that mono-ubiquitylation is sufficient to induce endocytosis and lysososmal degradation of RTKs in mammalian cells, as has previously been reported in yeast.[42] Other studies performed with yeast cells demonstrated that in addition to cargo ubiquitylation, the endocytic machinery is also regulated by ubiquitylation.[43] At the plasma membrane of mammalian cells, machinery's components include Eps15 and Epsin, adaptors sharing an UIM, and like in yeast, the E3 ligase involved in their ubiquitylation is a member of the Rsp5p/Nedd4 family. The activity of Nedd4 family ligases may be regulated by RTKs; Nedd4 and the Nedd4-like ligase AIP4 are phosphorylated upon EGF stimulation.[44] Another regulatory mechanism has been uncovered in flies: the deubiquitylating enzyme Fat Facets was shown to de-ubiquitylate Liquid Facets, an orthologue of Epsin,[45] which is associated with endocytosis in *Drosophila*.[46] A mammalian homologue of Fat Facets, FAM, was localized to multiple points of E-cadherin and Beta-catenin trafficking,[47] but its association with RTK endocytosis remains to be elucidated.

The Journey of RTKs to the Lysosome

Receptor endocytosis involves several distinct steps and a continuously decreasing gradient of intravesicular pH. Internalization requires clustering of cargo molecules over Clathrin-coated regions of the cell surface, membrane bending and formation of a Clathrin-coated vesicular structure. Following its formation and pinching off, the shuttle vesicle loses its coat and fuses with an apparently stationary compartment, the early endosome. The next sorting event occurs in the multi-vesicular body (MVB). However, unlike cargo sorting at the plasma membrane, receptors delivered to the limiting membrane of the MVB are sorted 'away from the cytoplasm', into invaginations and internal vesicles, which accumulate lysosomal enzymes.

Figure 1 schematically delineates the multi-step sorting process and Table 1 lists the respective protein players. This highly coordinated but incompletely understood process entails many protein-lipid and protein-protein interactions, which are depicted in Figure 2, and its default route targets receptors back to the cell surface (recycling). Early studies that compared the routes of endocytosis of wild type EGFR and a kinase-defective mutant concluded that internalization at the plasma membrane is largely a kinase-independent process, which is followed by efficient recycling.[48,49] This pathway is similar to the route taken by Transferrin receptors, which are constitutively internalized even in the absence of a ligand. Clathrin, along with its

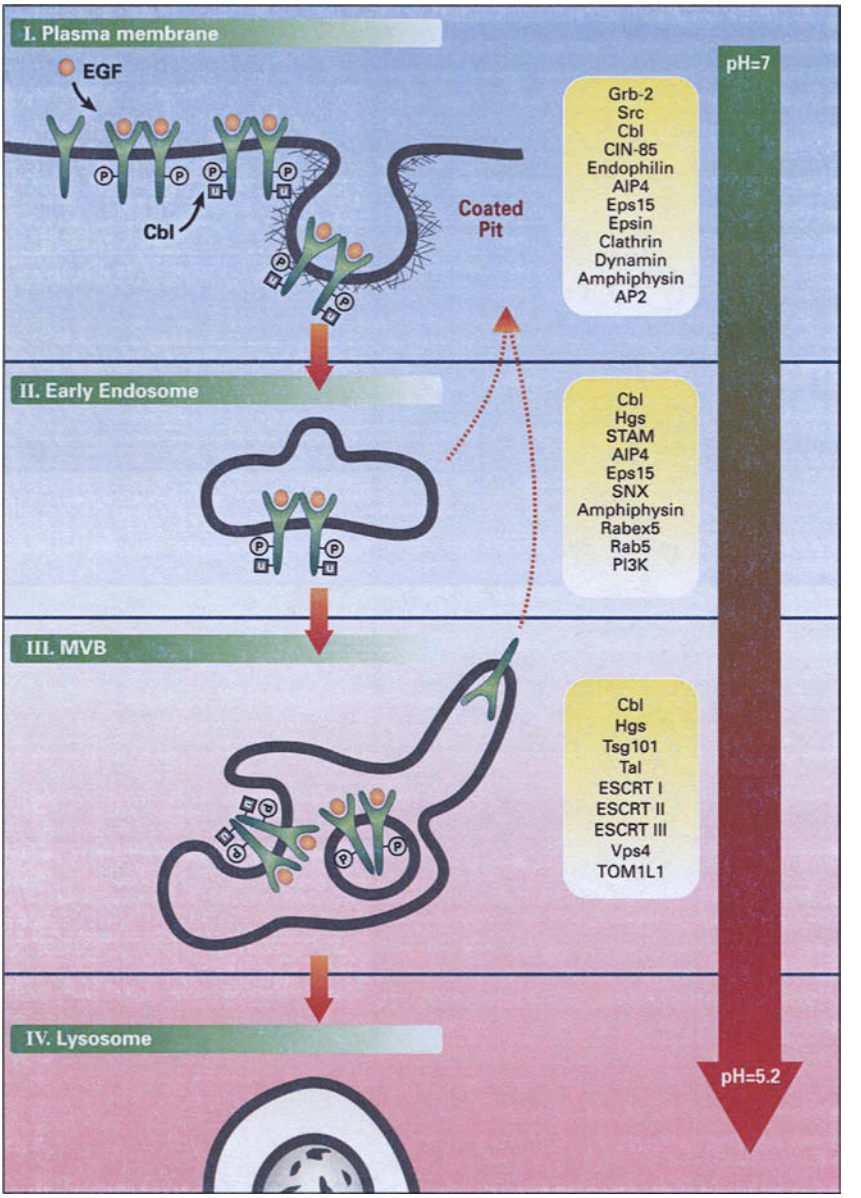

Figure 1. The journey of RTKs to the lysosome. Upon ligand binding at the plasma membrane, RTK molecules like EGFR cluster over Clathrin-coated pits, which subsequently pinch-off to form Clathrin-coated vesicles. The vesicle looses the Clathrin coat and fuses with the early endosome. Next, receptors are sorted to internal vesicles of the MVB and subsequently to lysosomes, where they undergo degradation by hydrolases. Alternatively, RTK molecules recycle back to the plasma membrane (dotted arrow). The major regulators of each sorting compartment are listed in the respective box, and the decreasing gradient of intravesicular pH is indicated by the vertical arrow. P letters refer to tyrosine phopshorylation and U letters refer to mono-ubiquitylation.

Table 1. Proteins involved in endocytosis of RTKs

Site	Species: Mammals	Yeast	Domains	Role
Membrane	EGFR	-	L/CR/JM/ TKD/ CT	RTK
	Src	-	Unique/SH3/SH2/TKD	Phosphorylates CHC and redistributes clathrin upon EGFR activation
	c-Cbl	-	TKB/RF/Pro/UBA	Ubiquitin E3 ligase
	AIP4	Rsp5p	C2/WW/HECT	Ubiquitin E3 ligase; interacts with Endophilin, Epsin, Eps15 and Cbl
	Epsin	Ent1/Ent2	ENTH/UIM/CBM/AEBM/NPF	Clathrin coat adaptor; AP2 binding protein
	Eps15	Ede1	EH/CC/UIM	Clathrin coat adaptor
	Endophilin	Rvs167	SH3/hinge region/NT/CC	Interacts with dynamin, AIP4 and CIN85; lipid modifying enzyme(LPAAT)
	CIN-85/SETA	Sla-1	SH3/Pro/CC	Adaptor protein; interacts with Cbl and endophilin; regulates clathrin mediated endocytosis
	Amphiphysin	Rvs161p/Rvs167p	BAR/Pro/CLAP/SH3	Recruits dynamin; interacts with AP2
Early endosome	Dynamin	Dynamin	GTPase/middle/PH/GED/PRD	Forms collars on invaginating pits
	Hgs	Vps27p	VHS/FYVE/UIM/Pro/CC/PG	Regulates endosomal sorting
	STAM	Hse-1	VHS/UIM/SH3/ITAM	Interacts with Hgs
	SNX	Vps5p/Vps17p/ YJL036W	PX/CC	Regulates intracellular sorting; interacts with Hgs and amphiphysin
MVB	Rabex	Vps9	GEF/CUE	Activates Rab5 and cooperates with other factors to promote endosome fusion
	Tal	-	LRR/ERM/CC/SAM/PTAP-PSAP/RF	Ubiquitylates Tsg101
	TOM1L1	ND	VHS/PTAP/GAT/GBD/SH3-BD/PBD /SH2-BD	Interacts with Hgs and Tsg101
	Tsg101	Vps23p	UEV/Pro/CC/SB	Recruits ubiquitylated proteins to ESCRTI

Table continued on next page

Table 1. Continued

Site	Species: Mammals	Yeast	Domains	Role
MVB	ESCRTI:			Involved in the sorting of ubiquitylated proteins
	Tsg101/	Vps23/	UEV/CC	
	Vps28/	Vps28/		
	Vps37c	Vps37	CC	
	ESCRTII:			Transiently recruited to endosomal membranes; required recruitment of ESCRTIII
	EAP25/	Vps25/	CC	
	EAP30/	Vps22/		
	EAP45	Vps36	NZF	
	ESCRTIII:			Polymerize to form intraluminal vesicles
	CHMP2/	Vps2/	CC	
	CHMP6/	Vps20/	CC	
	CHMP3/	Vps24/	CC	
	CHMP4	Vps32	CC	
	VPS4A,B	Vps4	CC/AAA	Dissociates ESCRT from the membrane de-ubiquitylating enzyme
	UBP	Doa4	CC/RD/CB/HB	

Protein regulators of endocytic sorting of RTKs like EGFR are listed according to their site of action. Note that the ubiquitin-specific processing protease (UBP) refers to UBPY/UBP8, which belongs to a family of mammalian ubiquitin hydrolases related to yeast Doa4p. Ubiquitin-binding proteins like Eps15, Epsin, Hgs and STAM are involved in ubiquitin-dependent regulation of receptor trafficking. The abbreviations used are: AEBM: alpha-ear binding motif; BAR: Bin-Amphiphysin-Rvsp; CB: cysteine box; CBM: Clathrin-binding motif; CC: coiled coil; CLAP: Clathrin-AP2 binding; CR: cysteine-rich; CT: carboxyl-terminal; CUE: Cue1p-homology; EH: Eps15 homology; ENTH: Epsin N-terminal homology; ERM: Ezrin-Radixin-Moesin; FYVE: Fab1/YOTB/Vac1/EEA1; GAT: GGA and Tom-1; GBD: Grb2 binding domain; GED: GTPase effector domain; GEF: guanine-nucleotide exchange factor; HB: histidine box; HECT: homologous to E6-AP C-terminus; ITAM: immunoreceptor tyrosine-based activation motif; JM: juxtamembrane; L: ligand binding; LPAAT: lysophosphatidic acid acyltransferase; LRR: leucine-rich repeats; NT: N-terminal alpha helical; PBD: p85 binding domain; PG: proline- and glutamine-rich; PH: pleckstrin homology; PRD: proline- and arginine-rich domain; Pro: proline-rich; PX: Phox; RF: ring finger; RD: rhodanese-homology domain; SAM: sterile alpha motif; SB: steadiness box; SH2: Src homology 2; SH2-BD: SH2 binding domain; SH3: Src homology 3; SH3-BD: SH3 binding domain; TKB: tyrosine kinase binding; TKD: tyrosine kinase domain; UBA: ubiquitin associated; UEV: ubiquitin E2 variant; UIM: ubiquitin interaction motif; Unique: a region which varies among family members; VHS: Vps27p/Hrs/STAM.

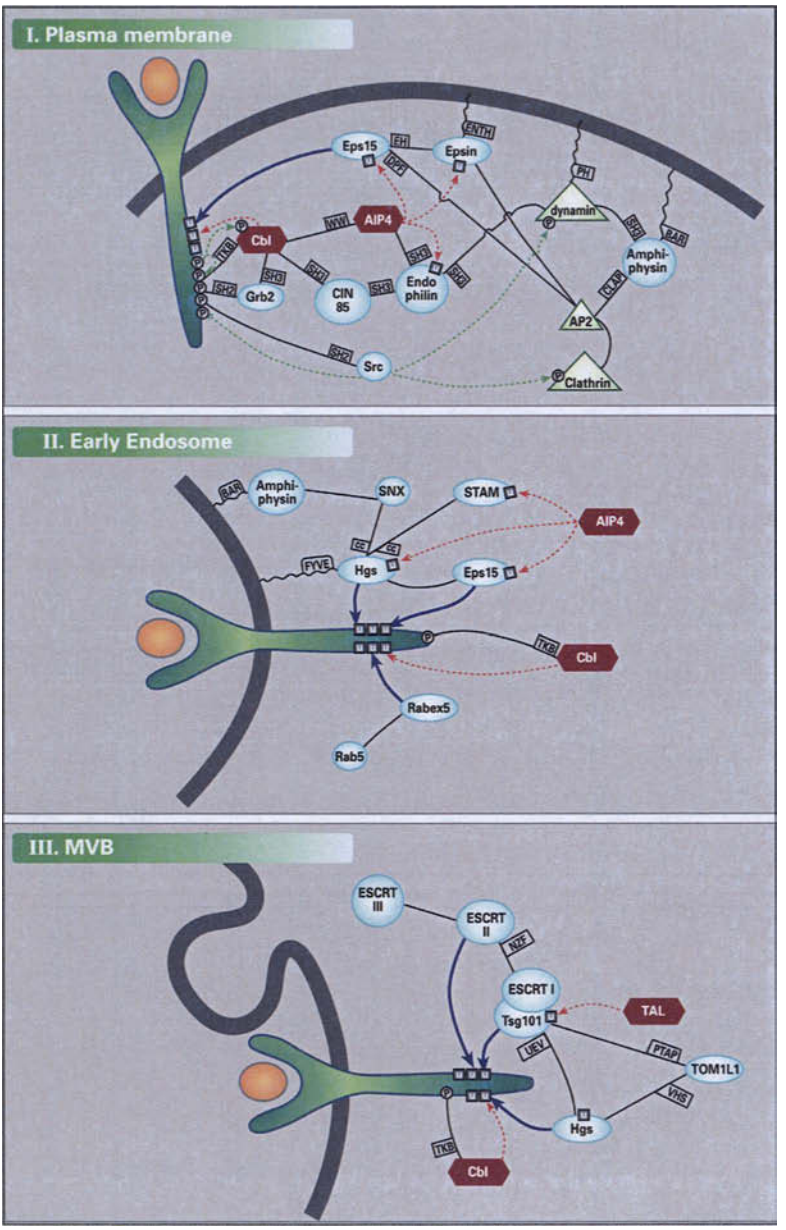

Figure 2. Protein networks involved in endocytosis of RTKs. Protein-protein and protein-lipid interactions, as well as phosphorylation and ubiquitylation of protein substrates, are illustrated for each step in the journey of an RTK like EGFR to the lysosome. Bold arrows (blue) indicate ubiquitin-mediated protein-protein interactions (ubiquitin-binding proteins are shown in ellipses). Dotted arrows refer to protein modifications, either phopshorylation (P; green) or ubiquitylation (U: red). E3 ligases are shown as hexagons. Some of the protein motifs involved in protein-protein interactions and protein-lipid binding (wavy lines) are shown in flags.

adaptor, AP2, and the GTPase Dynamin, play pivotal roles in the constitutive pathway of RTK endocytosis. To escape the recycling route, RTKs must recruit ubiquitin ligases, along with a group of mono-ubiquitin binding proteins (e.g., Eps15 and Epsin), which enable selective sorting of mono-ubiquitylated receptors, primarily at the early endosome and at the MVB.

Receptor Sorting at the Plasma Membrane

In the absence of ligands, a large fraction (40-60%) of EGFRs is found in caveolae, which are enriched in several signaling proteins, including Src. Random exit from the caveolae may be enhanced upon trans- or auto-phosphorylation of EGFR, primarily on tyrosine 845, recruitment and activation of Src.[50] Subsequently, Src phosphorylates both Dynamin[35] and the heavy chain of Clathrin, which is followed by Clathrin redistribution to the cell periphery[36] and enhanced endocytosis of EGFR.

The Clathrin-AP2-Amphiphysin Platform

Although the polyhedral lattice of Clathrin is the main component of budding vesicles, it offers only a mechanical scaffold incapable of selecting cargo. Sorting is carried out by several adaptors, the major one at the plasma membrane being the heterotetrameric complex AP2. AP2 binds specific cytoplasmic motifs of transmembrane cargoes, thus allowing their inclusion into coated pits. For example, in the case of EGFR, AP2 binds truncated receptor mutants through the motif ^{974}YRAL,[51] but alternative mechanisms seem to mediate endocytosis in the context of full-length receptors.[52] Another important function of AP2 is recruitment of Amphiphysins, ligands of Dynamin, which are anchored at the membrane through a BAR (Bin-Amphiphysin-Rvsp) domain. This domain senses, and in the case of Amphiphysin also imposes membrane curvature.[31] Further, during the budding process, Amphiphysin recruits Dynamin to the neck of the nascent vesicle, where Dynamin forms a ring-like collar that finalizes the fission process.

The Cargo Ubiquitylation Platform: Grb2-Cbl-CIN85

Ligand-induced activation of several RTKs leads to direct or indirect recruitment and phosphorylation of Cbl proteins,[53-55] whose orthologue in worms is a major negative regulator of EGFR signaling.[56] Cbl is an E3 ubiquitin ligase that binds tyrosine phosphorylated cargoes like EGFR through an amino-terminal SH2-like domain and recruits an ubiquitin-loaded E2 molecule to the RING domain. While it is clear that c-Cbl is recruited to the plasma membrane upon activation of EGFR[57-61] and internalization of EGFR is reduced when the interaction with c-Cbl is interrupted,[62,63] several reports suggest that receptor endocytosis may take place in the absence of an intact ubiquitylation machinery.[64] In addition to the E3 ligase activity, c-Cbl recruits a large number of protein partners, including Grb2 and CIN85. Both direct and indirect (via Grb2) recruitment of c-Cbl leads to receptor ubiquitylation.[63] However, these alternative modes may not be functionally redundant; Grb2 seems essential for receptor endocytosis[65] and its knockdown indicates an essential function in the initial steps of EGFR internalization.[59] This may reflect the ability of Grb2 to interact with CIN85, a scaffold molecule and an ubiquitylation substrate that recruits Endophilins to internalizing RTKs.[66,67] The SH3 domain of the Endophilin adaptor binds Dynamin, whereas the N-terminal region possesses a lysophosphatidic acid acyltransferase (LPAAT) activity, which may assist in curving the planar plasma membrane.

The Coat-Adaptor Ubiquitylation Platform: AIP4-Eps15-Epsin

Evidence in yeast indicates that ubiquitylation of components other than the cargo is required for receptor endocytosis: the E3 ligase Rsp5p ubiquitylates a component of the endocytic machinery prior to endocytosis of the membrane proteins Gap1 and Ste2p.[43,68] The mammalian orthologues of Rsp5p, AIP4 and Nedd4, mediate ubiquitylation of several coat adaptors sharing an ubiquitin-interacting motif (UIM). At the plasma membrane, these include Eps15 and Epsins.[69-74] Eps15 includes multiple protein-protein interaction modules; three Eps15

homology (EH) domains, which bind the NPF tripeptide motif, a homodimerization coiled-coil domain, multiple DPF tripeptide motifs, which bind to the Clathrin coat via AP2, and a tandem UIM. In addition to the UIM, Epsin harbors a $PI(4,5)P_2$ binding domain, the Epsin N-terminal homology (ENTH) domain, which plays a role in the initiation of the budding process.[32] Epsins are recruited to biological membranes by several additional interactions, which involve Clathrin, AP2 and Eps15. Upon EGF stimulation, the latter undergoes tyrosine phosphorylation, recruitment to the plasma membrane[75] and mono-ubiquitylation.[76] Several models have recently been proposed for how ubiquitin-binding motifs direct mono-ubiquitylation of coat-adaptors like Eps15. Accordingly, the UIM may bind ubiquitin in the thiol-ester intermediate state of E3 ubiquitin ligases.[77] Ubiquitin is then transferred to the UIM-containing protein, which no longer interacts with the E3 ligase and is not subject to poly-ubiquitylation. According to an alternative model, intramolecular interactions between the UIM and ubiquitin masks the lysine residue at position 48, the main site for ubiquitin branching, thus inhibiting further chain assembly.[78]

Receptor Sorting at the Early Endosome

Once sorted to Clathrin-coated vesicles, internalized receptors are delivered within 2-5 minutes to a tubular-vesicular network located in the cell periphery. The Clathrin-coated vesicle sheds Clathrin and fuses with an internal vesicle to form the early endosome. Endocytic vesicle maturation is concomitant with a reduction in the internal pH and accumulation of hydrolytic enzymes. This endosomal trafficking is controlled by a group of GTPases, primarily Rab5, which are regulated by EGFR. The early endosome is a major site for sorting of endocytosed cell surface receptors, which are to be recycled to the cell surface or destined for degradation in lysosomes (reviewed by ref. 79). Newly synthesized lysosomal hydrolases are also delivered to the lysosome from the trans-Golgi network via the endosome. At this organelle, ubiquitylation serves as a sorting signal for endocytosed receptors and newly synthesized lysosomal proteins to be incorporated into the luminal vesicles of the MVB.[80] Presumably, ubiquitin-mediated sorting of cargoes at the endosome shares some attributes with the process which occurs at the membrane, but a partly different set of UIM-containing adaptors and their direct partners, namely: Hrs/Hgs, STAM/East, Hbp and Sorting nexins (SNX), participate in endosomal sorting.[81]

The Role for Rab5

The Rab5 small GTPase plays a central role in the formation of endosomes by regulating Clathrin-coated vesicle formation, the fusion of endocytic vesicles, and their movements along microtubules (reviewed in ref. 82). Several studies have established that the activated EGFR modulates the GTPase activity of Rab5 by targeting either GTPase-activating proteins (GAPs), such as RN-Tre, or GTP exchange factors (GEFs), such as Rabex-5/Vps9p or RIN1.[83] A critical effector of Rab5 is Rabaptin-5, which forms a complex with Rabex-5. Upon activation of Rab5 by Rabex-5, the Rabaptin-Rabex complex induces its own membrane recruitment through Rabaptin-5. This positive feedback loop is thought to create a microenvironment enriched in active Rab5 on the membrane, where other Rab5 effectors are concentrated. The activation of Rab5 through EGFR and RIN1 stimulates both the internalization and degradation of activated EGFRs, as also occurs upon expression of constitutively active mutants of Rab5 (reviewed in ref. 84). Recently, two new protein partners for Rab5, APPL1 and APPL2, were discovered.[85] These proteins bind to the active, GTP-bound form of Rab5 and label a sub-population of peripheral endosomes to which a fraction of the internalized EGFR is targeted.

The Hgs-STAM-SNX Platform

Hrs/Hgs was originally characterized as a tyrosine phosphorylation substrate for several growth factor receptors, including EGFR. In addition to a single UIM, Hgs is composed of several recognizable domains: an amino-terminal VHS domain, the FYVE phosphatidylinositol 3-phosphate binding domain responsible for endosomal localization,[86] a proline-rich region,

and a coiled-coil domain, which recruits Sorting Nexin 1 (SNX-1). Apart from the phox-homology (PX) domain, through which SNX-1 binds PI3P and PI(3,5)P_2, SNX-1 contains a BAR domain, which endows the ability to form dimers and sense the high membranes curvature of early endosomes.[87] Similarly, SNX-16 directs sorting of EGFR to the endosomal compartment and regulates EGF-induced signaling.[88] Other ligands of the coiled-coil domain of Hgs are the signal transducing adaptor molecule (STAM/East) and the Hgs-binding protein (Hbp), both implicated in the regulation of growth factor receptor levels and signaling.[89,90] Through interactions with ubiquitylated cargo proteins on the early endosome via the VHS and UIM domains, STAM participates in the sorting of cargo proteins for trafficking to the lysosome.[91] Apparently, Hgs forms a multivalent complex with STAM and Eps15. The localization of this complex to Clathrin-rich regions of the endosome membrane is controlled by Vps4, a AAA-type ATPase, which has been implicated in MVB formation.[92]

The Tom-1 Platform

Tom-1 (target of Myb1) was identified as a protein whose expression is induced by a viral oncoprotein (v-Myb). The conserved amino-terminal domains of Tom-1 and its relative, Tom-1L1 (also referred to as Srcasm;[93]), harbor a VHS (Vps27p/Hrs/STAM) domain followed by a GAT (GGA and Tom-1) domain. The GAT domain of Tom-1 binds ubiquitin and Tollip (Toll-interacting protein) in a mutually exclusive manner. Interestingly, Tollip recruits Tom-1 and ubiquitylated proteins to the early endosome.[94] Thus like Hrs, Tom-1 is involved in sorting of ubiquitylated proteins into clathrin-coated microdoamins of early endosomes and the MVB.

Receptor Sorting at the MVB

During their maturation, early endosomes loose their tubular extensions, translocate along microtubules toward the nucleus and become more acidic. This process leads to the formation of the late endosome, a vesicular compartment that receives no direct transport of vesicles from the plasma membrane. Late endosomes are dynamic compartments with pleiomorphic organization, containing cisternal, tubular and vesicular regions with numerous membrane invaginations, which gave them the name multivesicular bodies (MVBs). This prelysosomal organelle is enriched in proteins targeted for degradation, and its limiting membrane contains high amounts of LAMP1, a characteristic protein of lysosomes. Fusion of the limiting membrane of the MVB with the lysosomal membrane results in the delivery of luminal vesicles and their contents to the hydrolytic interior of lysosomes, where they are degraded. Ubiquitin is thought to act as a positive signal for cargo sorting in the MVB. The corresponding components of the MVB sorting machinery were uncovered in large part by genetic analyses in yeast, which led to the identification of many proteins implicated in vacuolar protein sorting (called Vps; reviewed in ref. 95). Sorting of ubiquitylated proteins into the MVB pathway is executed by three distinct complexes of class E Vps proteins, called endosomal complexes required for transport (ESCRT-I, -II, and -III), and the AAA-type ATPase Vps4.

The ESCRT-I Platform

In yeast, the 350kDa ESCRT-I complex is composed of Vps23, Vps28 and Vps37, and it plays a crucial role in the selection of ubiquitylated cargoes.[96] The cargo-binding component is most likely Vps23p, which harbors a catalytically inactive ubiquitin conjugating-like (UBC-like) domain, also called ubiquitin E2 variant (UEV) domain. The mammalian orthologue of Vps23p, tumor susceptibility gene 101 (Tsg101), was originally isolated by using an anti-sense RNA screen for malignant transformation of murine fibroblasts.[97] Tsg101 has been shown to bind ubiquitin through the UEV domain, which includes a second binding site for a tetrad amino acid motif, P(T/S)AP.[98] When Tsg101 is ablated, internalized EGFR molecules are shunted from the degradative pathway to a recycling route that enhances and prolongs signaling.[99] This may contribute to the tumorigenic phenotype exhibited by fibroblasts in which tsg101 expression has been ablated. Tsg101 directly interacts with Hgs via a PSAP motif, which binds the

UEV domain of Tsg101.[100] Hgs-Tsg101 interactions are required for EGFR transport from the early to the late endosomes. Presumably, Hgs binds ubiquitylated cargo and recruits Tsg101, which then also interacts with ubiquitin moieties on the cargo and assembles ESCRT complexes for subsequent trafficking.[101] Tsg101 undergoes mono-ubiquitylation by an E3 ligase called Tsg101-associated ligase (Tal).[102] Upon ubiquitylation, Tsg101 is no longer capable of EGFR sorting, presumably because its own ubiquitins block cargo loading at the UEV. Thus cyclic ubiquitylation and de-ubiquitylation of Tsg101 by Tal and an unknown deubiquitylation enzyme may underlie ESCRT-1-mediated sorting of ubiquitylated cargoes into the lumen of the MVB in animal cells.

The ESCRT-II and ESCRT-III Platforms

The 155 kDa soluble ESCRT-II complex of yeast includes Vps22, Vps25, and Vps36. This protein complex transiently associates with the endosomal membrane and thereby initiates the formation of ESCRT-III. Like other sorting platforms, ESCRT-II contains an ubiquitin-binding subunit, Vps36, which binds ubiquitin via an NZF (Np14 zinc finger) domain.[103] In analogy to ESCRT-I, ESCRT-II selects and sort MVB cargoes for delivery to the lumen of the lysosome.[104] Removal of ubiquitin from MVB cargoes, which occurs in yeast cells before the cargo enters the luminal vesicles of an MVB, requires the enzymatic activity of a de-ubiquitylating enzyme called Doa4 (degradation of alpha-2), a homologue of certain mammalian ubiquitin hydrolases (e.g., UBPY). Doa4 recruitment to the endosome requires the correct assembly of ESCRT-III.[105] After ubiquitin removal, cargoes are sorted into invaginating vesicles that eventually bud into the lumen. This requires the function of additional regulators, which are currently unknown (reviewed in ref. 95). Finally, the disassembly and release of the entire MVB sorting machinery, which allows the ESCRT machinery to recycle back into the cytoplasm, is controlled by the AAA-type ATPase Vps4. Future studies will likely uncover the role of deubiquitylation enzymes and other mammalian proteins associated with MVB sorting. One interesting candidate is Tom-1L1.[106] The VHS domain of Tom-1L1 interacts with Hgs, while its PTAP motif is responsible for binding to Tsg101. In addition, Tom-1L1 possesses several tyrosine motifs at the C-terminal region that mediate interactions with members of Src family kinases and other signaling proteins, such as Grb2 and p85-PI3K. Thus Tom-1L1 recruitment to the sorting machinery of MVB may induce activation of signaling complexes.

The Interface of Receptor Trafficking and Signaling

In general, ligand-induced receptor endocytosis serves as a machinery that terminates growth factor signaling. Consistent with this notion, SLI-1, the single Cbl orthologue of *C. elegans*, is a major negative regulator of EGFR signaling,[107] and an internalization-defective mutant of EGFR is characterized by enhanced signaling.[63] The default recycling pathway enables prolonged signaling, which explains the oncogenic action of transforming mutants of c-Cbl.[62] The efficiency of recycling decreases as receptors reach late compartments of endocytosis. Nevertheless, this process, unlike sorting for degradation, does not require the intrinsic kinase activity.[108] The mechanisms affecting recycling are incompletely characterized. For example, threonine phosphorylation of EGFR by protein kinase C inhibits receptor ubiquitylation and enhances recycling,[109] but the underlying mechanism remains unknown. In the case of G protein-coupled receptors there are at least two recycling pathways, a direct (fast) pathway that depends on Rab4, and a slow recycling route mediated by both Rab4 and Rab5 (reviewed in ref. 110), but RTK recycling is less characterized. Nevertheless, it has been reported that Rab11 plays a role in late recycling of EGFR.[111] Accumulating evidence indicates that the internalized EGFR continues to bind and phosphorylate downstream signaling proteins in predegradative intracellular compartments, leading to activation of signaling pathways distinct from those originated at the cell surface (reviewed in ref. 112). Internalized EGFRs are enzymatically active, hyperphosphorylated and associated with Ras-GAP, Shc, Grb2 and mSOS.[113] Moreover, endosomal EGFR signaling is sufficient to activate the major signaling pathways leading to cell proliferation and survival, as well as suppression of apoptosis induced by serum withdrawal.[114]

Ligand-Independent Pathways of RTK Endocytosis

Accumulating evidence show that in addition to the well-characterized ligand-induced pathway of RTK down-regulation, there are alternative, ligand-independent mechanisms for receptor internalization and degradation. For example, anti-receptor antibodies cause relatively slow endocytosis and degradation of EGFR and ErbB-2/HER2.[115] Because certain monoclonal antibodies to these RTKs are clinically used to treat various types of cancer (reviewed in ref. 116), the mechanism of antibody-mediated endocytosis of RTKs is relevant to therapeutic applications. Due to their bivalence, most anti-receptor antibodies weakly activate tyrosine auto-phosphorylation and downstream signaling, including phosphorylation of c-Cbl. This may account for the ability of antibodies to elevate receptor ubiquitylation.[117] Nevertheless, antibodies can down regulate RTKs through a c-Cbl- and ubiquitylation-independent mechanism, whose rate is proportional to the size of antibody-receptor lattices formed at the cell surface.[118] Unlike anti-receptor antibodies, which interact directly with the internalizing receptor, agonists of G protein-coupled receptors, such as the beta$_2$-adrenergic receptor, indirectly act upon EGFR. These agonists induce dimerization and auto-phosphorylation of EGFR, followed by beta-arrestin-dependent internalization of both EGFR and the beta$_2$-adrenergic receptor.[119] Yet a third mechanism of ligand-independent endocytosis of RTKs is put into motion under stress conditions. These include osmotic stress, stimulation with the tumor necrosis factor and irradiation by ultraviolet (UV) light. Immunofluorescence microscopy demonstrated that upon UV treatment of cells, EGFR translocates to internal vesicles but undergoes no tyrosine phosphorylation, ubiquitylation or degradation.[120-122] On the other hand, oxidative stress induces extensive tyrosine phosphorylation of EGFR, but the phosphorylated receptor fails to recruit c-Cbl and the ubiquitylation machinery.[123] Clearly, ligand-mediated endocytosis of RTKs is not the only way to internalize active receptors and the intracellular routing is dictated by the type of stimulus. However, the mechanisms underlying the alternative routes of RTK endocytosis remain largely obscure.

Perspectives

Owing to the inherent dynamics and complexity of the endosomal system, our understanding of endocytosis and the signals that target receptor molecules to different intracellular pathways is still incomplete. The importance of in-depth understanding stems from the oncogenic potential of many members of the RTK family and the association of transforming ability with localization at the cell surface. Nevertheless, recent analyses of EGFR and several other receptors, such as the nerve growth factor receptor and the transforming growth factor-beta receptor, indicate that receptors stimulate distinct signaling pathways when residing in different sub-cellular compartments. Along with exhaustive identification of the respective molecular players and their post-translational modifications, sophisticated microscopic methods will likely contribute to future efforts to harness or manipulate endocytic pathways for clinical applications.

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CHAPTER 10

Endocytic Trafficking and Human Diseases

Rosa Puertollano*

Abstract

In the last several years an increasing number of genes associated with different human diseases has been identified. Interestingly, many of these genes have been demonstrated to encode components of the intracellular sorting machinery that mediates the selective trafficking of lipids and proteins in the secretory and endocytic pathways. This chapter highlights the molecular basis for selected diseases associated with defects in intracellular trafficking with a specific focus on disorders resulting from aberrant endosomal sorting.

Introduction

The endocytic pathway receives cargo from the cell surface via endocytosis, biosynthetic cargo from the late Golgi complex, and various molecules from the cytoplasm via autophagy. Recently, the intracellular trafficking machinery implicated in delivery of newly synthesized lysosomal proteins from the trans-Golgi network (TGN) to endosomes, fusion of lysosomes with late endosomes, and sorting of membrane proteins into luminal vesicles at endosomes have become topics at the forefront of the study of endosomal biology. The importance of these sorting events is reflected in the fact that defects in the endosomal transport machinery are implicated in a range of human diseases. In this chapter examples of the connection between alterations of the normal trafficking of lipids and proteins along the endosomal pathway and different human pathologies will be described. As criteria for inclusion in this discussion, the protein responsible for the disorder must be a component of the endosomal sorting machinery and the observed cellular defect must arise as a consequence of disturbances in a specific endosomal pathway. The diseases reviewed have been grouped according to the demonstrated or proposed function of the defective protein in intracellular transport (Table 1).

Defects in Protein-Sorting Machinery

One of the best characterized sorting events is the delivery of lysosomal hydrolases from the TGN to endosomes (Fig. 1). After synthesis at the endoplasmic reticulum (ER), lysosomal hydrolases are transported to the Golgi complex where they are posttranslationally modified by the addition of mannose 6-phosphate groups.[1] These groups are then recognized by specific transmembrane receptors named mannose 6-phosphate receptors (MPRs). MPRs contain sorting motifs in their cytosolic tail that allow them to interact with clathrin adaptors[2,3] and be recruited to clathrin-coated areas of the TGN, from which carrier vesicles deliver the MPR-hydrolase complexes to endosomes. The acidic pH within endosomes induces release of the hydrolases from the MPRs, allowing the receptors to return to the TGN for additional rounds of sorting. Fusion events between lysosomes and endosomes could

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Endosomes, edited by Ivan Dikic.

Table 1. Disorders associated with defects in endosomal trafficking

Disorder	Defective Protein	Protein Function
Defects in protein sorting machinery		
Mucolidosis II or I-cell disease	N-acetylglucosamine 1-phosphotransferase	Addition of mannose-6-phosphate groups to lysosomal enzymes
Hermansky-Pudlak syndrome	β3A subunit of AP3 or HPS	Lysosomal trafficking
Defects in lysosomal biogenesis		
Mucolipidosis IV	h-mucolipin-1	Ca^{2+} channel
Chediak-Higashi syndrome	CHS1	Lysosome fusion/fission
Danon disease	Lamp2	Regulation of autophagy and lysosomal stability
Defects in endosomal Rabs		
Griscelli syndrome	Rab27a	movement of melanosomes and cytotoxic T lymphocyte granule release
Charcot-Marie-Tooth type 2 Neuropathy	Rab7	late endocytic transport and lysosome biogenesis
Choroideremia	Rab escort protein (Rep1)	Geranyl transferase required for Post-translational processing of Rabs proteins
X-linked mental retardation	RabGDP-dissociation inhibitor (GDI)-α	Regulator of Rabs activation
Tuberous sclerosis	Tuberin	Rab5 GTPase activating protein
Defects in lipid trafficking		
Niemann-Pick type C	NPC1 and NPC2	Transport of cholesteryl ester from late endosomes to other organelles
Oculocerebrorenal syndrome of Lowe	inositol polyphosphate 5-phosphatase OCRL-1	conversion of phosphatidyl inositol (4, 5)biphosphate to phosphatidyl inositol 4-phosphate
Autoimmune diseases		
Antiphospholipid syndrome	Autoimmune disease against Lysobisphosphatidic acid	Formation of multivesicular bodies
Stiff-Man syndrome	Autoimmune disease against Amphiphysin I	Regulator of clathrin mediated endocytosis

mediate the final delivery of hydrolases to the lumen of lysosomes where they participate in the degradation of different substracts.

Mucolipidosis Type II or I-Cell Disease

Mucolipidosis type II or I-cell disease is an allelic disorder caused by a deficiency in uridine diphospho (UDP)-*N*-acetylglucosamine: *N*-acetylglucosaminyl-1-phosphotransferase, one of the enzymes that participate in the addition of mannose 6-phosphate to lysosomal hydrolases within the Golgi.[4] Symptoms associated with this disorder include multiple skeletal abnormalities (e.g., congenital hip dislocation and dwarfism), hepatosplenomegaly, and mental retardation.[5,6] The symptoms become obvious during infancy and may include multiple

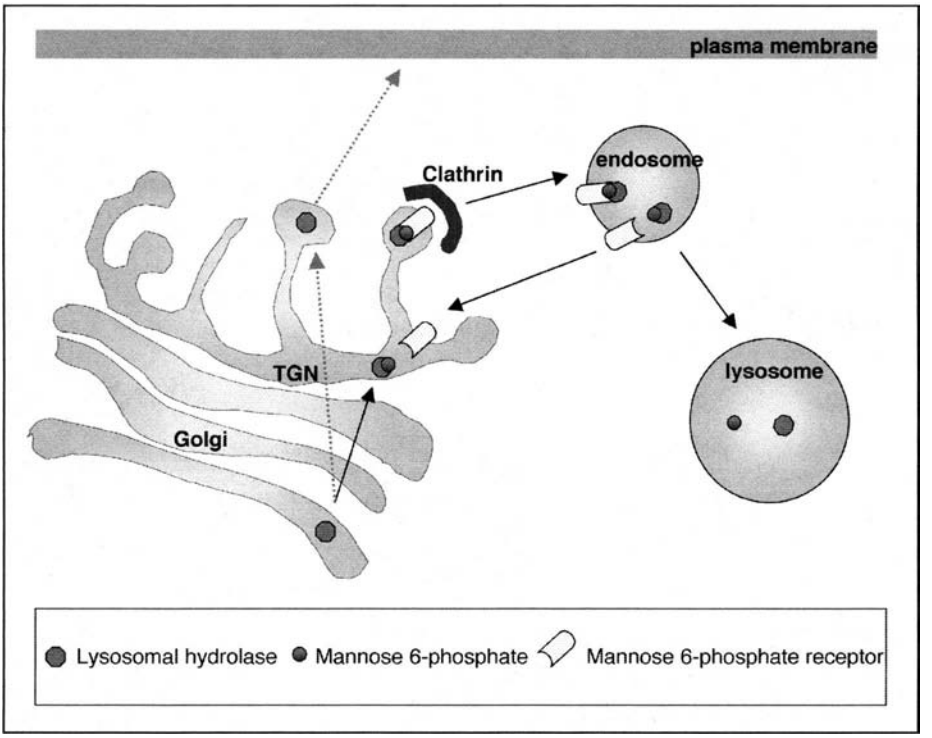

Figure 1. Sorting of lysosomal hydrolases. Addition of mannose 6 phosphate residues allows newly synthesized lysosomal hydrolases to interact with specific receptors called MPRs and be delivered to endosomes through a clathrin dependent pathway (black arrows). Once at endosomes hydrolases dissociate from MPRs and travel to lysosomes while receptors come back to the TGN and participate in additional rounds of transport. In patients with I-cell disease, the inability of hydrolases to receive mannose 6 phosphate residues prevents their interaction with MPRs. Enzymes cannot be transported to lysosomes and are instead secreted into the medium (hatched arrows).

abnormalities of the skull and face and growth delays. Early enzymologic studies showed that cultured fibroblasts from patients were deficient in a number of lysosomal enzymes. Furthermore, these enzymes were found to be present in excess in tissue culture media and in extracellular fluids, such as serum and urine.[7,8] In addition, patient fibroblasts present characteristic phase-dense intracytoplasmic inclusions originated by accumulation of undegraded products. These fibroblasts were named inclusion-cells or I-cells giving name to the disease. Importantly, some cell types, such as hepatocytes and Kupffer cells, seem to have a normal content of lysosomal enzymes despite the absence of phosphotransferase activity[9] indicating that alternative pathways for the trafficking of hydrolases may exist.

Hermansky-Pudlak Syndrome

Hermansky-Pudlak syndrome (HPS) includes a group of several autosomal recesive disorders characterized by oculocutaneous albinism, clotting defects, and storage of ceroid-like material resulting from abnormal function of lysosomes, platelet-dense granules, and melanosomes.[10,11] To date, mutations in seven human genes (HPS1-7) are known to induce HPS. Recent studies have revealed that most of these genes encode proteins that participate

in the formation of three different complexes termed lysosome-related organelles complex (BLOC)-1, -2, and –3.[12] It is thought that BLOCs regulate lysosomes and lysosomes-related organelles biogenesis, though they may also be involved in the movement and distribution of late endosomes and lysosomes within the cell.[13] In contrast, HPS-2 patients contain mutations in the β3A subunit of the heterotetrameric complex AP-3 (adaptor protein-3). Four AP complexes have been identified (AP-1, AP-2, AP-3, and AP-4) that regulate sorting at different cellular compartments through mediating the formation of coated transport vesicles, as well as the selection of vesicle cargo.[14] AP-3 is involved in the trafficking of lysosomal proteins from the TGN (and probably also early endosomes) to lysosomes, as evidenced by the fact that several lysosomal membrane proteins (lamp-1, lamp-2, CD63, and limp-II) are missorted to the cell surface in AP-3-deficient mammalian cells.[15,16] In addition, mice deficient in a β3B, an isoform that is exclusively expressed in brain, suffer from spontaneous epileptic seizures and display morphological abnormalities at synapses, suggesting that AP-3B might regulates the formation and function of a subset of synaptic vesicles in the brain.[17]

Defects in Lysosomal Biogenesis

In the last several years, numerous models have been proposed to explain how material targeted for lysosomal degradation is delivered from late endosomes to lysosomes.[18] For example, the endosomal maturation theory was updated to the kiss-and-run premise, in which multiple restricted fusion events would take place between late endosomes and lysosomes allowing delivery of luminal components while maintaining the integrity of the limiting membranes.[19] Alternatively, it has been proposed that a complete fusion between these two compartments might also occur, resulting in the formation of endosome-lysosome hybrid organelles. Lysosomes would be reformed from the hybrid in a process that requires recycling of membrane proteins to endosomes or the TGN and condensation of the intraluminal content. Interestingly, such hybrid organelles have been observed both in vivo and in vitro[20,21] and may in fact act as the major site for hydrolysis of endocytosed molecules. The machinery implicated in the fusion between late endosomes and lysosomes has not been characterized, but indirect evidences suggest that it could involve the small GTPase Rab 7;[22] a tethering complex formed by the mammalian homologs of the yeast proteins Vps11p, Vps16p, Vps18p, Vps33p, Vps39p, and Vps41p;[23,24] and specific SNAREs.[25] Calcium also seems to play a fundamental role for fusion and lysosomal reformation.[26,27]

Mucolipidosis Type IV

One of the disorders associated to defects in the late steps of endocytosis and lysosomal biogenesis is Mucolipidosis type IV (MLIV). MLIV is an autosomal recessive lysosome storage disorder characterized by severe psychomotor retardation and oftalmological abnormalities, including corneal opacity, retinal degeneration, and strabismus.[28] Patient's cells often contain enlarged vacuolar structures that accumulate sphingolipids, phospholipids and mucopolysacharides and display a higher pH than normal lysosomes. Interestingly, MCOLN1, the gene mutated in MLIV patients, encodes a protein termed h-mucolipin-1[29-31] that functions as a Ca^{2+} permeable channel and can be modulated by changes in pH and Ca^{2+} concentration.[32] It has been proposed that h-mucolipin-1 may be involved in the regulation of lysosomes biogenesis, and more specifically in the reformation of lysosomes from hybrid organelles. In agreement with this idea, recent experiments have shown that mutants of cup-5, the *C. elegans* orthologue of h-mucolipin-1, are associated with accumulation of endocytosed green fluorescent protein in enlarged vacuoles. Importantly these structures resemble endosome–lysosome hybrid organelles, based on the presence of RME-8 and LMP-1 (two distinct markers of late endosomes and lysosomes, respectively).[33,34]

Chediak-Higashi Syndrome

Chediak-Higashi syndrome is a disorder characterized by defects in blood clotting and pigmentation. However, in contrast with HPS, Chediak-Higashi patients also show neurologic dysfunctions and immunological deficits, including accumulation of large lysosomal granules in leukocytes, large eosinophilic, neutropenia, increased susceptibility to infection, and abnormal malignant lymphoma.[35] As a result, death often occurs before the age of seven years. The protein defective in this disorder is CHS1[36] and, although its function is still unknown, it has been reported that over expression of different CHS1 domains in Cos-7 cells causes dramatic changes in the size of lysosomes.[37] Since the fusion of secretory lysosomes with the plasma membrane is also inhibited in CHS1 defective cells, it was proposed that this protein probably regulates membrane fusion/fission events. This is in agreement with a recent study that describes an interaction between CHS1 and a soluble SNARE complex protein implicated in membrane fusion.[38]

Danon Disease

It has also been suggested that some lysosomal membrane glycoproteins (LPGs, that include lysosome-associated membrane proteins or LAMPs and lysosomal integral membrane proteins or LIMPs) could be implicated in lysosomal biogenesis.[39] These proteins are major components of the limiting membrane of lysosomes, but are also present in late endosomes. Based on their heavy glycosylation it was assumed that LPGs main function is to protect membranes from degradation by lysosomal hydrolases. LAMP-2 deficiency leads to Danon disease in humans, a pathology characterized by cardiomyopathy, myophaty and variable mental retardation.[40] Electron microscopy studies reveled a massive accumulation of authophagic vacuoles in numerous tissues, including hearth, muscle, and liver suggesting that LAMP-2 might play a role in autophagy and lysosomal stability. In addition, it has been reported that LIMP-2/LGP85 over expression causes accumulation of enlarged hybrid organelles while depletion of this protein in mice results in deafness, urogenital track obstruction, and peripheral neuropathy.[41]

Defects in Function of Endosomal Rabs

Rab proteins are small monomeric GTPases with molecular masses in the 20–30 kDa range. Multiple Rabs have been shown to participate in the formation, fusion and movement of vesicular traffic intermediaries between different membrane compartments of the cell.[42-45] Rabs function as molecular switches by cycling between two interconvertible forms, a cytosolic GDP-bound (inactive) form and a membrane associated GTP-bound (active) form. In addition, Rab proteins contain unique, hypervariable C-terminal domains with either one or, more frequently, two Cys residues both of which are modified by geranylgeranyl groups. This prenylation occurs in several steps and is essential for Rab association with intracellular membranes. Newly synthesized Rabs bind to a 95 kDa protein named Rab escort protein (REP) forming a stable complex that is then recognized by a Rab geranylgeranyl transferase (RabGGT), which catalyses the transference of geranylgeranyl groups to the Rabs C-terminal cysteines. Prenylated Rabs can be delivered to their appropriate donor membrane where specific Rab guanine nucleotide exchange factors (GEF) replace GDP with GTP (Fig. 2, steps 1-5). Active GTP-bound Rabs can now recruit different effectors and exert their specific functions that may include cargo selection and budding, as well as movement, tethering and fusion of vesicles at their final destinations. Finally, Rab GTPase activating proteins (GAP) promote GTP hydrolysis and render Rabs into an inactive GDP-bound conformation. Removal of inactive Rabs from the target membranes is mediated by Rab GDP-dissociation inhibitor (GDI) that can maintain Rabs in a GDP-bound inactive form in the cytosol or recycle them back to donor membranes (Fig. 2, steps 7-9).

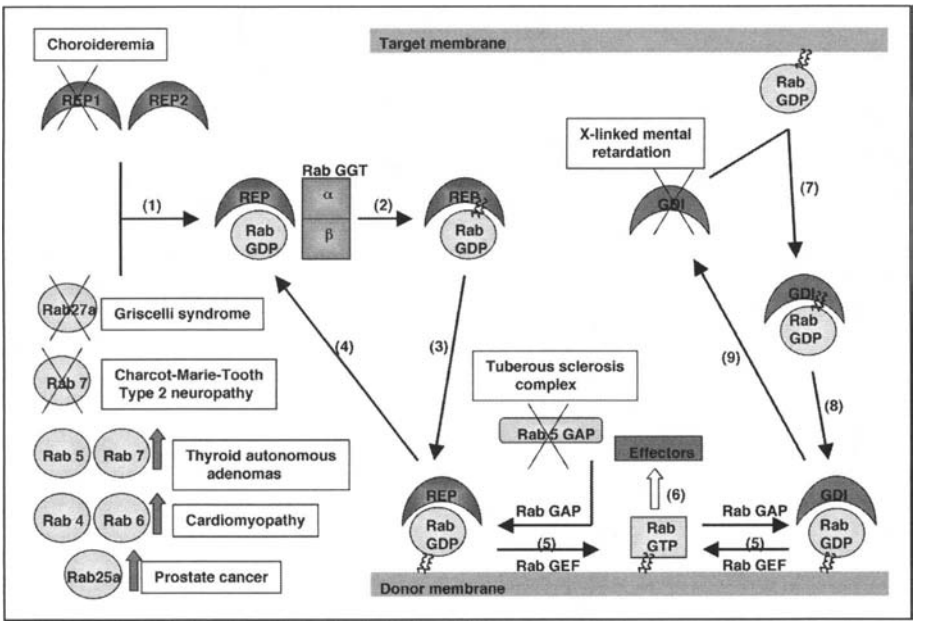

Figure 2. Diseases associated with defects in endosomal Rabs. After synthesis in the cytosol, Rab proteins form a stable complex with REP (1). RabGGT can recognize this complex and catalyze the transference of geranylgeranyl groups to the C-terminal region of Rabs (2). Prenylated Rabs are delivered to donor membranes (3) where specific GEFs promote the exchange of GDP by GTP (5) activating the Rabs that can now recruit effectors and exert their functions (6). In addition, GAP proteins stimulate GTP hydrolysis and render Rabs in a GDP-bound inactive state (5). RabGDI retrieves inactive Rab proteins from the target membrane and maintains them in an inactive state in the cytosol (7) or delivers them back to the donor membrane (8). Several human diseases caused by defects (cross) or over expression (gray arrows) of different Rabs and Rabs regulators are indicated.

Importantly, numerous pathologies have been associated with loss of function of Rabs and Rab regulators, as well as Rab overexpression. These are described below and in Figure 2.

Pathologies Associated with Loss of Function of Rabs and Rabs Regulators

Griscelli Syndrome

Griscelli syndrome is rare autosomal recessive disease induced by the absence of Rab27a.[46-48] In melanocytes Rab27a promotes the recruitment of myosin Va and melanophilin to the melanosome membrane, thus allowing interaction of melanosomes with the actin cytoskeleton. This interaction mediates the concentration of melanosomes in the tip of melanocyes dendrites and the transference of pigments to keratinocytes.[49-50] Absence of Rab27a causes a dramatic alteration of melanosomes trafficking, and accumulation of melanosomes in the central region of melanocytes, resulting in defects in hair and skin pigmentation and partial albinism.[51] In addition, defects in Rab27a expression also correlate with impaired immunological response due to defects in the secretion of lytic granules by cytotoxic T lymphocytes (CTLs).[52,53] In some patients neurological alterations has also been reported, although this is thought to be a secondary effect consequence of uncontrolled activation of T-lymphocytes and macrophages and subsequent leukocyte infiltration of the brain.[54]

Charcot-Marie-Tooth Type 2 Neuropathy

Mutations that disrupt Rab7 function are responsible for Charcot-Marie-Tooth type 2 neuropathy.[55] This disease is characterized by a severe motor and sensory neurons impairment, distal muscle weakeness and high frequency of foot ulcers that often require amputation because of recurrent infections. Interestingly, mutations in myotubularin-related protein 2, a phosphatase implicated in the metabolism of phosphatidylinositol (3,5) biphosphate and phosphatidylinositol 3-phosphate;[56] and mutations in KIF1B, a kinase that regulates synaptic transport,[57] also result in different forms of Charcot-Marie-Tooth disease, indicating that Rab7, myotubularin, and KIF1B may participate in the same pathway.

Choroideremia

Choroideremia (CHM) is a X-linked disease characterized by a slow degeneration of photorepectors, choriocapillaris, and retinal pigment ephitelium. This pathology usually starts in the second or third decade of life by progressive night blindness and loss of peripheral vision and often results in complete blindness.[58,59] CHM is caused by loss-of-function mutations in REP-1, a protein that regulates Rab prenylation (see above). However, it is thought that this mutation may be partially compensated by REP-2. This could explain why CHM phenotype is restricted to some very specific cells types and predicts the existence of retina-specific factors that interact with REP-1.

In mice reduction in the activity of RabGGT activity correlates with clotting disorders, including prolonged bleeding times, thrombocytopenia, and reduced platelet granule content, as well as with albinism and inability of CTLs to polarize lytic granules toward the immunological synapse with the target.

X-Linked Mental Retardation

Mutations in RabGDIα have been found in patients with X-linked mental retardation.[60] Mammalian Rab GDI consists of three members: Rab GDI α, β, and γ. Rab GDIα is specifically expressed in neuronal tissue where regulates the activity of Rabs implicated in neurotransmission, such as Rab3a.[61] Rab GDIα-deficient mice display neuronal hypersensitivity and high susceptibility to suffer epileptic seizures,[62] indicating that Rab GDIα might play an important role as a negative regulator of the synaptic function.

Tuberous Sclerosis Complex

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by the presence of benign tumors called hamartomas in multiple organs, including the central nervous system, lung, kidney, heart, and skin; learning and behavioral difficulties; and renal complications.[63] Mutation of two genes, TSC1 and TSC2, result in this clinical disorder. Importantly, TSC2 gene encodes tuberin, a GAP protein that stimulates GTP hydrolysis on Rab5,[64] suggesting that aberrations in the Rab5 dependent endocytic pathway might be linked to this disease.

Pathologies Associated to Rabs Overexpression

Alterations in the level of expression of a variety of Rabs have also been related to several human diseases. In some cases the overexpression may be consequence of somatic rearrangements while in other cases they are a consequence of sustained intracellular signaling. High levels of Rab5 and Rab7 have been associated with a certain type of benign thyroid tumors called thyroid autonomous adenomas (AA). It appears that this overexpression promotes an increased association of Rab5 and Rab7 with endosomes, resulting in augmented processing of thyroglobulin and thyroid hormone production.[65] Rab 7 over expression has also been observed in a mouse model for atherogenesis;[66] while the level of Rab 25, a Rab implicated in recycling to the apical membrane, is altered in prostate cancer cell lines.[67] It has also been noted that the levels of Rab6, which is involved in microtubule dependent pathways through the

Golgi apparatus and from endosomes to Golgi, as well as the levels of the endosomal Rab4 are upregulated in a dilated cardiomyopathy model overexpressing β2-adrenergic receptors.[68] Finally, increased expression of a Rab5 GAP named PRC17 has been reported in metastatic prostate tumors, indicating that alterations in the levels of Rab regulators may also account for a number of human diseases.[69]

Defects in Lipid Trafficking

Niemann-Pick Type C Disease

Cholesterol is a major structural component of mammalian cellular membranes and also acts as a regulator of lipogenic gene expression and membrane protein function. Cells have to carefully regulate the amount and distribution of cholesterol to assure proper function. The principal disorder associated with defects in cholesterol trafficking at endosomes is Niemann-Pick type C (NPC) disease.[70] This syndrome is characterized by progressive neurological degeneration and is often associated to hepatosplenomegaly. Patients usually appear normal for the first two years of life with symptoms appearing between 2 and 4 years. At the cellular level there is a clear accumulation of abnormal amounts of cholesterol and other lipids (including glycosphingolipids (GSLs), sphingomyelin, lysobisphosphatidic acid (LBPA), and phospholipids) in a late endosomal/lysosomal compartment. It is interesting to note that in neurons the total amount of cholesterol is not significantly different between NPC patients and controls, however, its distribution is dramatically altered. Cholesterol accumulates in enlarged endosomal/lysosomal structures located in the neurons cell body while it is absent from endosomal organelles at the distal axon.

95% of NPC patients contain mutations in NPC1, a multi-spanning transmembrane protein that localizes to late endosomes/lysosomes and is thought to regulate the trafficking of free cholesterol from late endosomes to other cell compartments, including the plasma membrane and endoplasmic reticulum (ER).[71,72] The remaining 5% of NPC patients is defective on NPC2, a soluble cholesterol binding protein that normally cycles between TGN and endosomes through a CI-MPR dependent pathway, but accumulates in late endosomes in NPC patients.[73] It is thought that accumulation of cholesterol in late endosomes resulting from mutations in either NPC1 or NPC2 could result in a "lipid traffic jam" that would trap other lipids and transmembrane proteins and disturb the cellular cholesterol homeostatic response. Recent experiments have shown that over expression of Rab 7 or Rab 9, but not Rab11, corrects defective lipid trafficking and abrogates cholesterol storage in NPC cells.[74] The mechanism that mediates this restoration if unknown though it has been suggested that Rab 7 and Rab 9 over expression could increase the transport of accumulated lipids from late endosomes.

Recently, some authors have pointed out similarities between NPC and Alzheimer's disease (AD).[75] These similarities include endosome enlargement, elevated hydrolase content, and accumulation of β-cleaved amyloid precursor protein (APP) and Aβ peptides within endosomes, and suggest that defects on endosomal trafficking might lead to defective processing of APP and neurodegeneration.

Oculocerebrorenal Syndrome of Lowe

Another pathology associated with defects in lipid trafficking is the oculocerebrorenal syndrome of Lowe (OCRL), a rare X linked disorder characterized by mental retardation, congenital cataracts, and reduced ammonia production by the kidney.[76] The gene mutated in this syndrome encodes a phosphatidylinositol (4, 5) biphosphate 5-phosphatase named OCRL-1 that catalyzes the conversion of phosphatidylinositol (4, 5) biphosphate to phosphatidylinositol 4-phosphate.[77] Fibroblasts from patients with Lowe syndrome showed abnormalities of the actin cytoskeleton as well as atypical distribution of gensolin and

alpha-actinin, two actin-binding proteins regulated by calcium and phosphatidylinositol (4, 5) biphosphate.[78] Actin is necessary for formation and maintenance of tight junctions. Since these structures play a crucial role in the function of renal proximal tubule and lens differentiation it has been proposed that defects in actin polymerization could account for the OCRL phenotype. However, it is important to note that phosphatidylinositol 4-phosphate also participates in the recruitment of clathrin adaptors to Golgi membranes. It has been described that depletion of a major phosphatidylinositol 4-kinase by siRNA causes AP-1 to become citosolic, an effect that can be reverted by adding exogenous phosphatidylinositol 4-phosphate.[79] As mentioned above, clathrin adaptors mediate transport of different proteins, including lysosomal hydrolases, from TGN to endosomes, therefore, defects in OCRL-1 could disrupt the normal endosomal trafficking.

Autoimmune Diseases

Autoantibodies have traditionally been used as diagnostic markers for various autoimmune diseases. Interestingly, some of these autoantibodies recognize endosomal proteins and their presence correlates with specific disorders. For example, autoantibodies to early endosomal antigen (EEA1), a protein located on the cytosolic face of early endosomes and implicated in regulation of endosome fusion,[80,81] have been reported in the sera of patients with neurological disorders.[82,83] Early endosomes are key functional components of both presynaptic and post-synaptic neurons indicating that the presence of autoantibodies against EEA1 could induce aberrations in the endosomal pathway and defects in neurotransmission.

The antiphospholipid syndrome (APS) is a disorder in which antibodies binding to phospholipids (PL) are thought to be involved in the development of thrombosis and/or pregnancy complications.[84] It has been described that the sera of some of these patients contain antibodies against lysobisphosphatidic acid (LBPA), a lipid highly enriched in late endosomes that regulates the formation of multivesicular bodies (MVBs).[85,86] Moreover, binding of specific antibodies to LBPA induces an alteration in the structure and function of late endosomes.[87]

There are other examples of autoimmune diseases associated with the presence of antibodies directed against endosomal proteins. Stiff-Man syndrome, a rare central nervous system disorder characterized by muscular rigidity and episodic spasms[88] correlates in a subset of patients with the presence of anti-amphiphysin autoantibodies;[89] necrotizing and crescentic glomerulonephritis (NCGN) is frequently associated with circulating autoantibodies against Lamp-2;[90] and CLIP-170, a protein that connect endosomes with cytosolic microtubules,[91] has been identified as a new autoantigen in three patients suffering from limited scleroderma, glioblastoma and idiopathic pleural effusion.[92]

Conclusions and Future Prospects

The recent identification of new components of the sorting machinery has allowed a better understanding of the molecular mechanism of different human diseases. At the same time the identification of disorders caused by defects in intracellular trafficking provides very valuable information about the role of specific proteins in a particular pathway. Some of the examples included in this chapter show how defects in different proteins can cause similar phenotypic effects. In addition, the presence of components with redundant functions may mask the defect of a protein or limit the effect of this mutation to specific cell types. Certainly, a better comprehension of the mechanisms that regulate intracellular trafficking will help to design improved strategies to fight numerous human diseases.

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CHAPTER 11

Endosomes—Key Components in Viral Entry and Replication

Mark Marsh*

Abstract

Endosomes play key roles in the cellular infection cycles of many viruses. Initially implicated in virus entry, recent research has demonstrated that endosomes can also be required at other stages in viral replication. Endosomes can provide platforms for viral nucleic acid replication and virus assembly, or play roles in modulating anti-viral immune responses. To these ends viruses exploit various attributes of endosomes such as the low luminal pH, unique trafficking properties, cellular location and composition. In turn, viruses have become remarkable tools for analysing endosome function.

Introduction

Endosomes were initially described as intermediates between the plasma membrane and lysosomes in the endocytic transport pathway.[1] Increasingly, these organelles are being recognised as key regulators of endocytic membrane traffic, protein sorting and signalling and they play essential roles in a multitude of cellular functions. Endosomes also have key functions in the activities of many pathogens and toxins. For viruses, endosomes were initially implicated in entry, but recent research has found that endosomes can also function at other stages in viral replication. Endosomes can provide platforms for viral nucleic acid replication and virus assembly, or play roles in modulating anti-viral immune responses. To these ends, viruses exploit the properties of endosomes, including the low luminal pH, trafficking functions, cellular location and composition. In turn, viruses have become remarkable tools for analysing endosome function. I will discuss a number of these activities focussing primarily on the roles of endosomes in virus entry and assembly.

Virus Entry

Prior to the early 1980's, electron microscopic (EM) studies had suggested a role for endocytosis in the cellular entry and infection by a number of viruses.[2] However, knowledge of the pathways involved was at best rudimentary and the molecular mechanisms were not understood. Insights to how endocytosis and endosomes could be used in the entry of cell-free viruses initially came from work on several enveloped viruses, especially Semliki Forest virus (SFV).[3] Indeed one of the original lines of evidence that lead to the description of endosomes as discrete components of the endocytic pathway was from studies of SFV entry.[4] Importantly, this work established that viral endocytosis is not just a cell's attempt to send a potentially

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harmful agent to lysosomes, but is an essential part of the mechanism used by many viruses to infect cells. These conclusions were reached by applying a range of experimental approaches including biochemistry, morphology, infectivity and pharmacological approaches to attempt to understand virus entry. Much of this work has been reviewed extensively.[5-8]

Cell-free viruses protect their genetic material within a protein shell (nonenveloped viruses), or a protein shell surrounded by a lipid membrane (enveloped viruses). To infect a cell, a virus must dock to receptors on the surface of the target cell, release its DNA or RNA from within the coat (uncoating) and transfer it across a limiting membrane of the cell to the cytoplasm (penetration). Uncoating and penetration are usually tightly coupled to ensure that the viral nucleic acid is only released when there is also the potential for it to reach the host cell cytoplasm.

Three types of mechanism to regulate uncoating and penetration have been identified to date. These can operate on both enveloped and nonenveloped viruses and two require the functional activities of endosomes. In the first, docking of the viruses to specific receptors can trigger uncoating and penetration, i.e., the receptors act as keys to unlock the virus. One of the best-characterised examples of this is the entry of the human immunodeficiency virus (HIV), an enveloped retrovirus. HIV binds to a binary receptor complex of CD4 together with CCR5 or CXCR4 (seven transmembrane domain G protein-coupled receptors for chemokines). Binding of the viral envelope protein (Env) to CD4 initiates conformational changes in Env that generate a second binding site specific for the chemokine receptor. Binding to the chemokine receptor initiates further conformational changes in Env that expose the fusion peptide in the gp41 (transmembrane) subunit of Env, resulting in fusion of the viral membrane with the target cell plasma membrane (see ref. 9). The fusion event simultaneously releases the viral RNA-containing core from within the virion to the cytoplasm. A similar type of receptor-driven uncoating/penetration reaction is believed to occur for poliovirus, a nonenveloped picornavirus. In this case, binding of the poliovirus receptor (PVR/CD155) to 'canyons' that surround protrusions on the surface of the virus particle loosens the interactions between the protein subunits of the virus capsid. This allows the internal capsid protein VP4 and the N-terminus of VP1 to be exposed on the virus surface and insert into the target cell membrane. This is believed to result in the formation of a pore through which the viral RNA is delivered to the cytoplasm.[10,11] Poliovirus and HIV bind their receptors at the cell surface, and penetration probably occurs at the plasma membrane. However for other picornaviruses and retroviruses (and perhaps for poliovirus as well), penetration may also occur in endosomes following endocytosis from the cell surface.[12-14] Indeed the endosomal route may offer some distinct advantages (see below).

The second mechanism for regulating uncoating and penetration is via pH changes. Rather that being receptor-driven, the events leading to the fusion of enveloped viruses or uncoating/penetration of nonenveloped viruses are triggered by exposure of the virions to mildly acidic pH. One of the best-characterised examples of this type of mechanism is the enveloped orthomyxovirus, influenza. The fusogenic envelope protein of influenza virus is the haemmagglutinin (HA). HA binds specifically to sialic acid-containing plasma membrane glycoproteins and glycolipids, which mediate endocytosis of intact virus particles. Following delivery to endosomes, the low pH triggers conformational changes in HA that expose the fusion peptide and initiate membrane fusion (reviewed in ref. 15). Many other enveloped viruses also undergo pH-dependent fusion.[15] In addition, as with the receptor-driven reactions, pH-dependent uncoating and penetration can occur with nonenveloped viruses, including the adenoviruses and some picornaviruses. However, the molecular mechanisms involved in the penetration of these viruses remain to be established in detail. The dependency of these viruses on exposure to low pH requires that they undergo endocytosis to reach acidic early and/or late endosomes.

A third mechanism for regulating entry has emerged from studies of nonenveloped mammalian reoviruses. Following endocytosis of reovirus particles, endosomal cathepsins L and B

cleave the σ3 and μ1/μ1C outer-capsid proteins to generate infectious sub-viral particles capable of mediating penetration (reviewed by ref. 16). A similar type of mechanism also appears to apply for the enveloped filovirus, Ebola. Entry is dependent on membrane fusion catalysed by the viral envelope glycoprotein (GP). Earlier work established that Ebola virus is pH-dependent for entry[17,18] but, in contrast to viruses such as influenza and SFV, exposure to low pH does not induce GP membrane fusion activity.[19] It appears that cleavage of GP by a pH-dependent endosomal proteases, probably cathepsin B, or to some extent cathepsin L, is required to trigger fusion.[20] Again, the pH-dependency and proteolytic requirements of this virus require that it must undergo endocytosis for infection.

For acid-dependent viruses, endosomes play an essential role in entry and productive infection. Thus agents that raise the pH of endosomes (weak bases, carboxylic ionophores and specific inhibitors of the vacuolar H^+-ATPase) can inhibit entry and productive infection.[21,22] Depending on the pH required to trigger fusion, different endosome sub-compartments may be used. Hence wt SFV, which undergoes fusion at ~pH 6.2 enters cells from early endosomes,[23,24] whereas strains of influenza, which fuse at ~pH 5.5, penetrate from late endosomes.[25,26] In the absence of endocytosis and delivery to these compartments, infection does not occur. Although essential for the entry of acid-dependent viruses, endosomes may also be used by pH-independent viruses that do not require exposure to acidic pH. These viruses can be internalised by endocytosis and, assuming that the molecular components necessary to trigger the fusion/penetration reactions are present in endosomes, these viruses may also use the endosomal entry route.

Virus Endocytosis

SFV is internalised very efficiently through clathrin-coated vesicles (CCVs) and is delivered from these vesicles to early endosomes.[3,4,27] Experiments using antibodies and dominant negative constructs targeted at proteins involved in clathrin-mediated endocytosis (e.g., dynamin, Eps-15), indicated that clathrin-mediated endocytosis is essential for infection by SFV and related viruses.[28-30] However, these and similar studies also indicated that not all pH-dependent viruses use CCVs. Influenza virus, for example, can undergo endocytosis through CCVs, but inhibition of clathrin-mediated endocytosis does not significantly diminish infectivity, indicating that a clathrin-independent endocytic mechanism can also mediate internalisation of this virus.[30] At least three distinct clathrin-independent pathways have now been identified using different enveloped and nonenveloped viruses, at least two of these pathways appear to involve lipid-raft dependent processes and one of these involves the protein caveolin.[8] This latter pathway is especially important for the entry of simian virus 40 (SV40), a pH-independent, nonenveloped, polyomavirus,[31] though a raft-dependent pathway can also mediate uptake of this virus in caveolin-1 negative cells.[32] These experiments illuminate the multiplicity and versatility of endocytic mechanisms in animal cells and the ability of viruses to capitalise on these activities.

Caveosomes—Parallel Endosome-like Organelles

Following uptake in caveolae, SV40 particles are delivered to endosome-like organelles that have been termed caveosomes. SV40 does not undergo penetration in caveosomes but is sorted into membrane-bound tubules that transfer the virus to the endoplasmic reticulum where, through processes that are not currently understood, it is transferred to the cytoplasm and subsequently to nuclear pores, through which it enters the nucleus.[31] Caveosomes appear not to undergo acidification, to have stable caveolin-coated membrane domains and high cholesterol content. They also appear to be able to undergo a Rab5-dependent interaction with conventional endosomes that may be visualised readily in cells over-expressing dominant active Rab5.[33] In addition to providing a route for virus entry, these organelles have been implicated in the cycling and turnover of lipid raft domains and membrane components enriched in lipid rafts, in particular GPI-linked proteins. However, their precise function remains unclear.[34] For

the remainder of this review the term 'endosomes' will be used to describe the acidic, prelysosomal, organelles that receive membrane and content from CCVs.

Endosome Function in Virus Entry

The endosomal route of entry is frequently considered purely a mechanism to provide acid-dependent viruses with a portal into the cell. However, the endocytic pathway leads to the noxious environment of the lysosome, so why have some viruses evolved to use this risky route? Use of endocytic mechanisms ensures that viruses only infect viable cells with a functional endocytic pathway (and not mammalian red blood cells, for example) and allows the virus to control when and where it undergoes penetration. Uptake in endocytic vesicles may allow virus particles to pass through the cortical cytoskeleton that supports the plasma membrane,[35] and transport within endosomes may deliver viruses to specific cellular sites that are crucial for their replication. The unassisted movement of large molecular complexes in the cytoplasm is inefficient. Endosomes can recruit motor proteins such as dynein and move on cytoplasmic microtubules towards the microtubule organising centre (MTOC).[36] Viruses can exploit this activity to hitch a ride to the centre of the cell without the need to carry their own mechanisms for interacting with transport machineries.[37] Thus viruses that replicate in the nucleus, such as influenza and adenovirus, may infect cells more efficiently when carried to the perinuclear region in endosomes. By requiring the more acidic conditions found in late endosomes, viruses may bias their chances of fusing or penetrating from endosomes positioned close to the nucleus.[26] These types of transport events may be especially important for some infection events in vivo, where cell architecture tends to be more developed than in many culture models.

For pH-dependent enveloped viruses, the fusion events that lead to productive infection have usually been considered to occur at the limiting membrane of endosomes. Conceptually, fusion could also occur with the internal membranes of multivesicular endosomes, but there has been little indication that such fusion events could lead to productive infection. Recent studies, primarily with the rhabdovirus vesicular stomatitis virus (VSV), have suggested that such a pathway may be used in some circumstances.[38] This notion stems from experiments in which interference with transport from early to late endosomes appeared to inhibit virus entry, even though virus could be shown to fuse in the early endosome compartment. One interpretation of these studies was that viral fusion occurred with the internal vesicles in multivesicular endosomes (also called multivesicular bodies [MVB]), however, the viral RNA does not enter the cytoplasm until these internal vesicles reach late endosomes where they may fuse with the limiting membrane. Currently, it remains unclear whether this mode of entry is peculiar to the tissue culture model, why even in this model virus should prefer to fuse with the internal membranes and how the fusion of vesicles back to the limiting membrane occurs. However, this pathway resembles a mechanism proposed for the entry of anthrax toxin into cells, where again interaction with the internal membranes of MVBs appears to be crucial.[39] Moreover, influenza virus penetration from late endosomes appears to be blocked when cycling of the ESCRT machinery (see below) is inhibited, suggesting that normal MVB formation and function is also required for penetration by this virus.[40]

For alphaviruses, the limiting membranes of endosomes and lysosomes provide platforms for RNA replication. EM studies of alphavirus-infected cells show characteristic vacuolar structures termed 'cytopathic vacuoles' (CPV) similar in size to endosomes and lysosomes. The prominent feature of these CPV is that their surface is lined with 50 nm diameter vesicular invaginations, or spherules, that project into the lumen of the vacuole. These structures have been shown to be sites of viral RNA synthesis and to contain viral nonstructural proteins (reviewed by ref. 41). The spherules might be formed directly following endosomal fusion of incoming viruses, but similar structures are seen in cells transfected with viral genomic RNA, arguing that there is also some capacity for viral components to target endosome/lysosome membranes following their synthesis. The assembly of replication sites may provide a structural

framework to enhance RNA synthesis and/or provide some protection from innate defence mechanisms that target viral RNA.[41]

Endosomes in Virus Assembly

The endocytic pathway provides a mechanism for regulating the cell surface levels of many membrane proteins, in particular signal transducing surface receptors that influence cell division, differentiation and function. In a number of well-studied cases it appears that, following agonist binding and activation, mono-ubiquitination of the cytoplasmic domain of a receptor molecule by a specific ubiquitin (Ub)-E3 ligase leads to internalisation and sorting of the protein to late endosomes and lysosomes. A key step in this pathway occurs in endosomes, where the ubiquitinated proteins are recognised by a set of protein complexes, termed the ESCRTs (endosomal sorting complex required for transport) (reviewed in refs. 42,43), that sort the Ub-tagged proteins into the membrane vesicles that bud into the lumen of endosomes or MVB. A key recent discovery is that this ESCRT machinery is also required for the topologically related process of enveloped virus assembly. Currently, four genera of RNA viruses including retroviruses, rhabdoviruses, filoviruses and arenaviruses (e.g., Lasser fever virus) have been found to require the ESCRT machinery for key steps in their assembly.[42]

The role of the ESCRTs has been most intensively studied for retroviruses. For these viruses, assembly and budding is directed primarily by Gag, the polyprotein precursor of the matrix, capsid and nucleocapsid proteins. Retroviral Gag proteins all contain so-called late domains (L-domains) in which specific amino acid motifs are required for completing the budding reactions.[44-46] To date three distinct types of L-domain motif have been identified, exemplified by (i) the PTAP motif in the HIV Gag L-domain (p6), (ii) the PPxY motif in murine leukaemia virus (MLV) and (iii) the YxxL motif in an equine lentivirus, equine infectious anaemia virus (EIAV) (reviewed by ref. 42). Although quite distinct, these motifs can be moved within the Gag sequence and can be swapped between viruses without compromising assembly, suggesting they have similar functional activities. Significantly, each of the motifs has been found to interact with distinct ESCRT components. The PTAP motif mimics a PSAP sequence in the cellular protein Hrs, which recruits ESCRT-1 to endosomes through interaction with Tsg-101.[47,48] PPxY motifs are consensus binding signals for WW domain containing proteins. Recent studies indicate that MLV Gag binds specifically to WWP1, WWP2 and ITCH, a subgroup of WW and HECT domain-containing, Nedd4-related E3 ligases.[49] These proteins have been implicated in the ubiquitination events described above and may interact with ESCRT complexes.[50,51] Finally, the YxxL motif interacts with AIP1/ALIX,[52] another ESCRT-associated protein that has recently been implicated in the inward invagination of membrane vesicles into endosomes.[53] Thus, L-domain motifs can recruit ESCRT components for interaction with Gag assemblies.

HIV normally buds at the surface of infected T cells. However, the requirement for ESCRT complexes in HIV assembly, together with data indicating that HIV and SIV contain highly conserved endocytosis signals that limit expression of Env at the cell surface and target Env to endocytic organelles, prompted us to investigate whether HIV may in some situation bud into endosomes.[54-56] HIV particles have occasionally been observed within intracellular vesicles particularly in macrophages.[57] However, the nature of these vesicles and the biological significance of the intracellular pool of virus had not been examined. Recent studies have shown that in primary human monocyte-derived macrophages, HIV assembles primarily on endosomal membranes and that virus accumulates within these organelles (Fig. 1).[58,59] Biochemical analyses of the membrane protein composition of HIV particles released into the medium of these cells show that most of the infectious virus derives from endosomes.[58] This intracellular assembly contrasts with HIV budding at the plasma membrane of T cells, and it remains unclear how viral components are targeted differently in different cellular backgrounds. One possibility is that the kinetics of membrane traffic through the endocytic system differs between macrophages and T cells, with the result that the viral components required to form infectious virions accumulate at the plasma membrane in T cells but on endosomes in

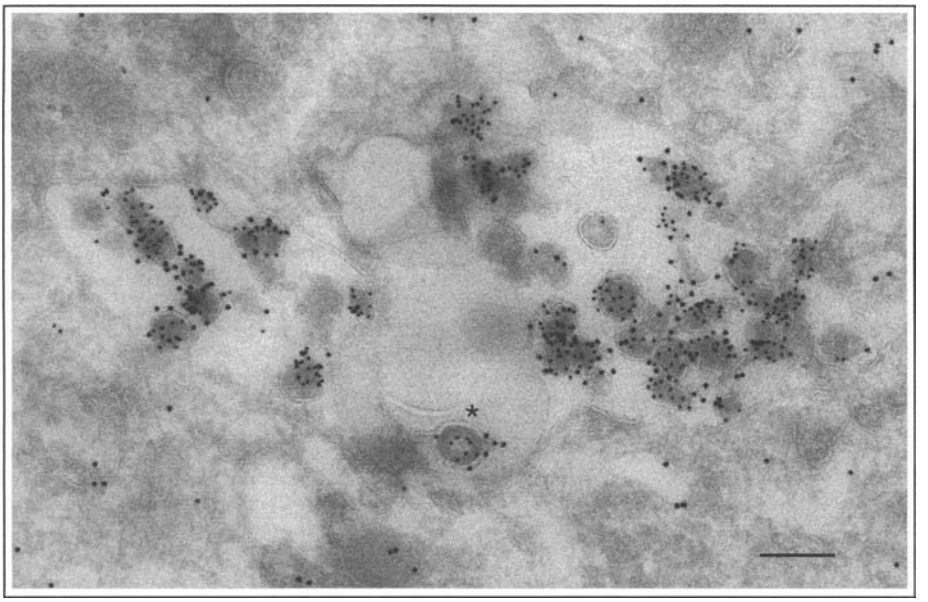

Figure 1. HIV assembly in macrophage endosomes. Human monocyte-derived macrophages were infected with HIV-$1_{Ba\text{-}L}$ for 7 days. The cells were then fixed, and ultrathin cryosections prepared for immunolabelling. The figure shows HIV particles within an endosomal vesicle. The virus particles are identified with antibodies against the viral capsid protein (p24) and 10 nm protein A gold. The sections were also labeled for the cellular antigen CD63 (15 nm protein A gold). CD63 can be seen on virus particles, on other membranes in the endosome and on neighboring vesicles. The asterisk identifies an immature virion in which the Gag protein has not yet undergone proteolytic cleavage. Scale bar = 200 nm.

macrophages. Although most (if not all) HIV appears to assemble intracellularly in macrophages, this virus can nevertheless be released from cells apparently through a secretory mechanism in which virus-containing endosomes fuse with the plasma membrane.[58,59] In some respects this discharge of virus has features in common with the release of the late endosomal/lysosomal cytotoxic granules of CD8 T cells. However, it remains to be seen whether the release of HIV-containing endosomes is regulated (see below).

One of the most prominent cellular proteins incorporated into HIV particles in macrophages is the tetraspanin CD63. This antigen can be seen on viruses in endosomes and is present in the membrane of viruses released into the macrophage medium.[58] CD63 traffics over the plasma membrane and is internalised through a C terminal YxxØ type signal but, in many cells, a major fraction of CD63 is seen in late endosomes and is frequently associated with the internal membranes of MVB.[60] The presence of CD63, together with other late endosomal antigens including LAMP-1 and to a lesser extent LAMP-2 has led to the suggestion that HIV might use late endosomes for assembly in macrophages. Significantly, this compartment is also the MHC class II compartment (see below). However, studies with other tetraspanins have identified another endosomal compartment distinct from the CD63-containing late endosomes that may be the site for virus assembly. The properties of this compartment appear to be modified by the virus infection, most notably by the acquisition of CD63. The exact nature of this novel endosome compartment remains to be established. However, initial studies suggest it may be related to an endocytic compartment in dendritic cells (DC) in which HIV can be sequestered during the process of trans infection of T cells (see below). Thus, for HIV in macrophages, budding into endosomes may have multiple advantages, enabling the virus to

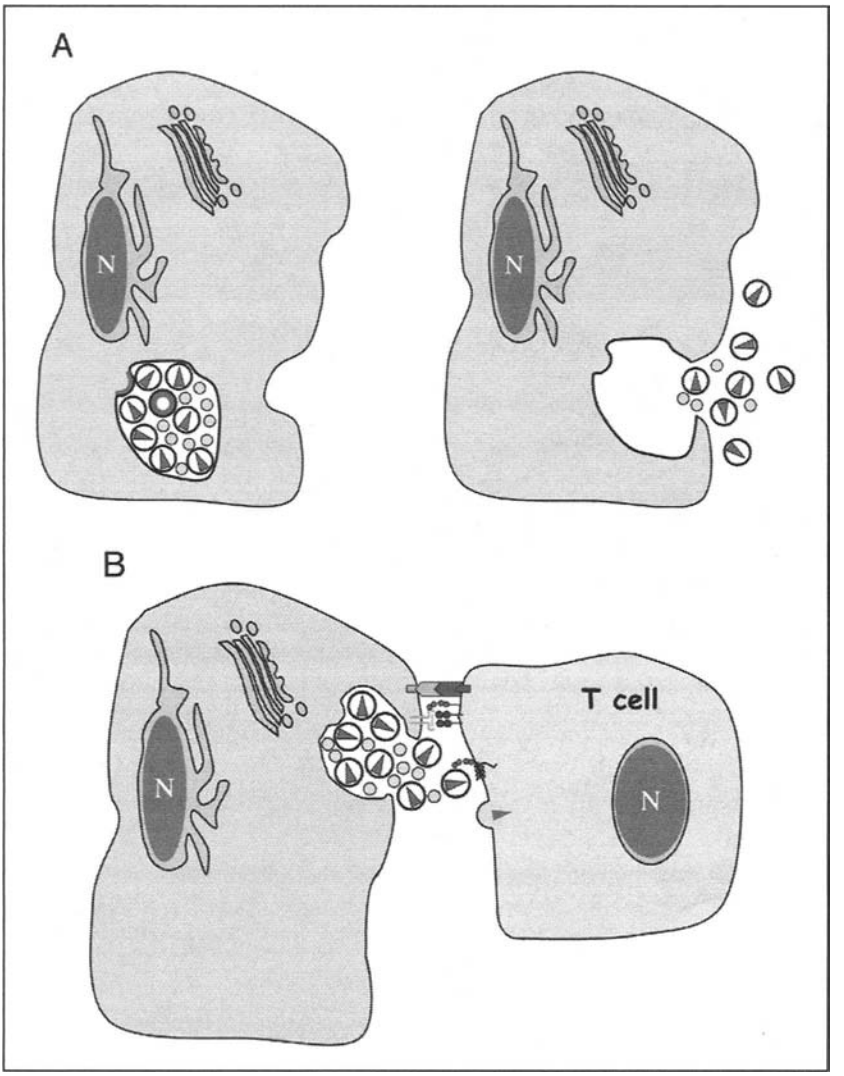

Figure 2. Model for HIV transmission between infected macrophages to T cells. In infected macrophages HIV assembles primarily in an endosomal compartment. This compartment has the capacity to translocate to, and fuse with, the plasma membrane releasing the cargo of viruses (Panel A). Interaction between infected macrophages and T cells may target the virus-containing endosomes to the zones of interaction, which then function as infectious synapses (Panel B). Similar events are proposed to occur when dendritic cells that have acquired virus by endocytosis interact with T cells.

assemble within a sheltered environment hidden from the attentions of the immune system, and allowing virus release to be coupled to the interaction of macrophages with $CD4^{+ve}$ T cells—a major host cell for the virus (Fig. 2).[61,62]

Assembly in endosomes appears not to be unique to HIV, as other retroviruses have been seen to bud into endosomes.[63,64] The recycling activities of the endosomal system are required to complete the assembly and release of Mason-Pfizer monkey virus, a B type retrovirus.[65]

Filoviruses have also been reported to require endosomal trafficking to target viral components to the cell surface for assembly,[66] and the β herpesvirus, human cytomegalovirus (HCMV), can bud into MVB and smaller vesicles and membrane cisternae related to late endosomes/MVB.[67] As with HIV, CD63 can be found in the HCMV envelope and, given that this virus can infect macrophages, the possibility exists that it has also evolved to exploit the endocytic system to facilitate its cell-to-cell transfer.

Endosomes in Antigen Presentation

Endosomes play a key role in antigen acquisition, processing and presentation on major histocompatibility type II (MHC-II) antigens. Dendritic cells (DC) patrol surface tissues to monitor for infection. These cells are particularly potent antigen presenting cells (APC) able to capture and present antigens to T cells. In immature DC (iDC), MHC-II antigens are stored within endosomal populations, the so-called MHC II compartment, or MIICs, in a nonpeptide bound form. EM shows this intracellular MHC-II to be located on vesicles within the MIICs.[68] However, when iDC are exposed to antigen and/or activation signals, such as bacterial lipopolysaccharide (LPS), they undergo a maturation programme during which the cells migrate to lymphoid organs and MHC-II molecules are loaded with peptide antigens and presented on the cell surface. During this process the proteins are loaded with antigen that can then be presented to CD4 positive T cells. Viral antigens derived from exogenous viruses taken up by endocytosis can be presented through this mechanism. Such a process may be particularly relevant to HIV where, as discussed above, budding may occur into endosomes. Indeed, in HIV-infected APCs, presentation of HIV antigens to HIV-specific T cells may result in efficient infection of these cells, and may account for the preferential loss of HIV-specific CD4 positive T cells early in the course of infection and abrogation of HIV-directed immune responses.[69]

Endosomal compartments in APCs may also play a role in disseminating viruses. For HIV, DCs are able to transmit virus to $CD4^{+ve}$ T cells without themselves becoming infected. This process of trans infection was first reported by Steinman et al who found that the most effective way of infecting T cells with HIV in culture was to present this virus in the context of a DC.[70] Recent experiments have begun to shed some light on the mechanisms underlying these events. APCs express a range of receptors able to bind and internalise biochemically distinct antigens. One group of these receptors is the C type lectins with specificity for high mannose oligosaccharides. Several of these lectins, in particular DC-SIGN (dendritic cell-specific ICAM-3 grabbing nonintegrin), can bind HIV Env without triggering fusion or infection.[71] The bound viruses are internalised, in part by clathrin-mediated endocytosis, and delivered to an endosomal compartment with properties distinct from conventional early and late endosomes.[72,73] Morphological observations indicate these organelles have internal membranes, i.e., they are multivesicular, and they contain a number of tetraspannins including CD9 and CD81, similar to the macrophage compartment where HIV assembles. When DC are then incubated with T cells, the virus-containing vesicles migrate to the zone of interaction between the DC and T cell and virus is transferred to the T cell, which becomes efficiently infected.[73,74] The zone of DC-T cell interaction through which virus transmission occurs has properties in common with immunological synapses and has been termed the 'infectious' or 'virological' synapse. These synapses may represent a mechanism that viruses have developed to exploit the special signalling activities of immunological synapses to facilitate their transfer from infected to uninfected cells. It is possible that HIV targets a special endosome compartment in APC (in particular macrophages and DC) either by endocytic uptake, or by assembly if the cell is infected, and that this compartment is targeted to the APC-T cell synapses.

Infectious synapses have been seen between infected and uninfected T cells for HIV, and for HTLV-1, where roles for endosomes are unclear. But the fact that a number of viral pathogens, including SIV,[75] HCMV,[76] hepatitis C virus[77] and Ebola virus,[78] interact with DC through DC-SIGN and/or other C-type lectins, suggest that a number of viruses may traffic through DC endosomes to enhance infection.

Viral Modulation of Endosome Trafficking

Viruses also exploit endocytic membrane trafficking pathways to modulate the distribution of cellular proteins important for pathogenesis. For example, in HIV infected cells plasma membrane CD4 is down-modulated by the viral Nef protein. This small myristoylated peripheral membrane protein can act as a linker to couple proteins that may not normally interact. Nef can induce the association of CD4 with the clathrin AP2 adaptor complex to bring about its uptake from the cell surface. In endosomes, Nef also appears to link CD4 to an endosomal complement of the COP-1 coat proteins thereby directing internalised CD4 molecules to lysosomes.[79,80] Similar mechanisms may regulate the levels of other cell surface proteins in HIV infected cells, though the modulation of major histocompatibility type I (MHC-I) antigens in HIV infected T cells appears to involve retargeting of newly synthesised MHC-I through Nef interaction with AP1 adaptors during transport through the exocytic pathway.[81]

Many viruses are able to down-modulate MHC-I expression to abrogate detection of infected cells by cytotoxic T lymphocytes. In a number of cases this involves perturbation of MHC-I synthesis but in others endocytic trafficking is required. KSHV, a γ herpesvirus linked to Karposi's sarcoma, appears to use mono-ubiquitin to target MHC-I antigens to lysosomes. This virus encodes two E3 ligases (modulator of immune recognition [MIR]1 and MIR2) that mono-ubiquitinate MHC-I heavy chains to induce their internalisation and sorting through endosomes to lysosomes (see above).[82] Other antigens, including ICAM-1, may be modulated by a similar mechanism. By contrast, the murine cytomegalovirus uses the gp48 protein encoded by the early gene m06 to bind newly synthesised murine MHC-I and, through an endosomal di-leucine trafficking motif, target the MHC-I antigens to endosomes and lysosomes for degradation.[83] HCMV employs ER retention mechanisms to reduce MHC-I cell surface expression, but this virus may use additional mechanisms to hide infected cells. As with many other herpesviruses, HCMV encodes seven transmembrane domain proteins that share features with chemokine receptors. One such protein is US28, which binds a range of CC and CXC chemokines, as well as the unusually membrane-bound CX_3C chemokine—fractalkine. By contrast to many cellular chemokine receptors, US28 is constitutively active for endocytosis and recycling[84] and may function as a chemokine sink, removing these chemoattractants from the environment around infected cells by endocytosis.[85]

As detailed studies of different viruses continue, it is likely that other mechanisms will emerge through which alteration in protein trafficking through endosomes play a role in modulating the expression of MHC and other cellular antigens.

Conclusions

In one way and another, endosomes have been exploited by a large number of viruses to facilitate their replication. The role of endosomes in the entry of many enveloped and nonenveloped viruses is now well established, although in many cases the details of these events still have to be worked out. For many RNA viruses, endosomes can provide platforms for the replication of viral nucleic acid and endosomes may be manipulated by the virus to provide a sanctuary in which replication can occur in isolation from cellular defence mechanisms. The nature of the immune response to viruses can also be manipulated through exploiting the properties of endosomes. Finally, and perhaps most surprisingly, some viruses appear to use the endocytic system for assembly. Given the close links between endosomes and lysosomes, this might seem to be a risky strategy but also suggests it has significant benefits for the virus. It may be that endosomal assembly is a strategy used most effectively in APC, where the endocytic pathway is modified to facilitate antigen presentation, and the viruses can exploit these properties to enhance their cell-to-cell transmission.

Over the last 20 years, work with viral systems has provided excellent tools for analysing the properties of endosomes. There is still much to learn and it is likely that viruses will continue to

provide insights into the functions of these organelles. Moreover, it is likely that further novel mechanisms through which viruses exploit the properties of endosomes to facilitate their replication and transmission will be discovered.

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CHAPTER 12

Toxins in the Endosomes

Núria Reig and F. Gisou van der Goot*

Abstract

Many bacteria owe their virulence to the production of protein toxins. These proteins usually play an important role in permitting the bacteria to successfully spread in the host and cause infection. With the exception of poreforming toxins and lipases, all toxins need to be endocytosed by the host cell to perform their toxin action. In the recent years, the increased knowledge of how toxins enter the cells and reach the cytoplasm have highlighted their ability to exploit, in its finest details, the membrane-trafficking systems of their hosts. In this chapter, based on selected examples we will review how toxins use the host endosomal system to reach their targets.

Introduction

In order to successfully colonize their host, many bacterial pathogens produce protein toxins. These are secreted proteins that modify the behavior of target mammalian cells. Whereas some act at the plasma membrane, such as pore-forming toxins or lipases, most are enzymes with cytoplasmic targets. This implies that they must cross a biological membrane to reach this intracellular milieu. To avoid deleterious membrane permeabilization, toxins never cross the plasma membrane. Instead, they are taken up by cells, transported to intracellular organelles, where membrane translocation occurs. We will here focus on the entry routes of toxins and their trafficking through the endocytic pathway. Five examples have been chosen to illustrate the different ways in which the endocytic pathway can be utilized or altered by toxins: cholera toxin (CT), produced by *Vibrio cholerae* and responsible for the secretory diarrhea associated with cholera disease,[1] Shiga toxin (ST), produced by *Shigella dysenteriae* and responsible for the vascular damage observed in shigellosis,[2] diphtheria toxin (DT), produced by *Corynebacterium diphteriae* and able to kill the intoxicated cells by inhibiting protein synthesis,[3] anthrax toxin, produced by *Bacillus anthracis*, the causative agent of anthrax[9] and Helicobacter vacuolating toxin (vacA), produced by *Helicobacter pylori*, a common colonizer of the human stomach and a risk factor for the development of peptic ulcer disease and gastric adenocarcinoma.[4] All five toxins, with the possible exception of VacA, are formed by 2 subunits, an A subunit that bares the enzymatic activity and a B subunit that has the ability to interact with the host cell and escorts the A subunit to its final destination (Table 1). We will only focus on the interaction of these AB toxins with the endocytic pathways, leaving out important interactions with the biosynthetic pathway for CT and ST and with mitochondria for vacA.

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Endosomes, edited by Ivan Dikic.

Table 1.

Toxin	Receptor	Structure	Activity	Target
Anthrax toxin	TEM8, CMG2	3 independent polypeptide chains: EF, LF, PA PA becomes heptameric after contact with the host cell	EF: calmodulin dependent adenylate cyclase, LF: metalloprotease	LF: MAPKKs
Cholera toxin	GM1	2 independant polypeptide chains: A and B, B being pentameric: A-B5	ADP-ribosyltransferase	G-proteins
Diphtheria toxin	HB-EGF	1 single polypeptide chain, with 2 disulfide linked subunits	ADP-ribosyltransferase	EF-2
Shiga toxin	Gb3	2 independant polypeptide chains: A and B, B being pentameric: A-B5	N-glycosidase	28S rRNA
VacA	Unknown	1 single polypeptide chain, with 2 subunits, becomes hexa or heptameric after contact with the host cell	channel forming	plasma membare, late endosomes, mitochondria

Different Portals of Entry into the Cell

Binding of bacterial toxins to targets cells is mediated by the specific interaction of the toxin B subunit, with a cell surface molecule that can be either a sugar, a lipid or a protein (Table 1). The receptor for CT is the ganglioside GM1,[1] for ST the ganglioside Gb3,[2] for DT the heparin-binding epidermal growth factor precursor (HB-EGF)[5] and for anthrax toxin two homologous receptors have been identified, Tumor endothelial marker 8 (TEM8)[6] and Capillary Morphogenesis gene 2 (CMG2).[7] VacA, in contrast to most toxins, appears to bind to a variety of different receptors including Protein-tyrosine phosphatase alpha, various lipids, the epidermal growth factor receptor and heparan sulphate[8] (for review see ref. 4).

Toxin B subunits can be either part of the same polypeptide chain as the A subunit, such as for DT and VacA, or synthesized separately by the bacterium, as for anthrax toxin, CT and ST. In addition, B subunits can be monomeric (DT) or multimeric (pentamers for CT and ST, heptamers for anthrax and VacA). These multimers are formed either during initial folding of the toxin (CT and ST) or at the surface of the target cell (anthrax toxin and VacA). This is of importance for understanding the endocytic routing of these toxins. Preformed multimers are indeed multivalent ligands, thus preferentially targeting regions of the plasma membrane where receptors are clustered. For example CT preferentially binds to caveolae, which contain clusters of GM1. In contrast, B subunits that are produced as monomers by the bacterium are monovalent ligands that, once bound to the cell surface, trigger clustering of the receptors upon oligomerization. This can in turn lead to receptor relocalization as observed for the B subunit of anthrax toxin, which is called the protective antigen.[9,10] It was indeed observed that heptamerization of the protective antigen leads to the redistribution of the receptor from the glycerophospholipidic region of the plasma membrane to specialized lipid domains rich in cholesterol and glycosphingolipids called lipid rafts.[11] Interestingly, four out of the five toxins

used as examples in this review associate with lipid rafts during the processes of cell binding and endocytosis: CT,[12] ST,[2] anthrax toxin[11] and VacA.[13] Our own unpublished observations show that DT does not associate with lipid rafts, using detergent resistance as a read out. It was however observed that cells lacking sphingolipids, an important component of lipid rafts as well as other membrane domains, are more sensitive to DT.[14]

Once bound to their respective receptors, toxins are internalized. As described in the previous chapters and recent reviews,[15-17] different pathways, which exist in parallel, allow entry into mammalian cells. These include the well-characterized clathrin-dependent pathway, the more recently characterized caveolar pathway as well as nonclathrin and noncaveolar pathways. As one might expect from opportunistic ligands such as toxins, each of these pathways has been hijacked, and usually one toxin can enter the cell by more than one pathway.

Toxin Entry by Clathrin-Mediated Endocytosis

Of the five example toxins discussed in this review, only vacA appears to be excluded from this pathway.[18] In contrast, for both diphtheria toxin and anthrax toxin, entry via clathrin-coated pits is the preferential route.[11,19,20] Interestingly however, whereas DT enters through constitutive endocytosis of its transmembrane receptor, the anthrax toxin *triggers* receptor uptake, via poorly characterized mechanisms that involve raft association[11] and most probably signaling events. For both toxins, targeting to the clathrin dependent entry route is most likely dependent on motifs in the cytoplasmic tails of their respective receptors, but this has not been analyzed. Surprisingly, ST and CT also can enter cells via clathrin-coated pits despite the fact that they bind to gangliosides, which being in the outer leaflet of the membrane can not interact with cytosolic sorting machineries.[21-26] As for the anthrax toxin, association to lipid rafts might trigger signaling to the endocytic machinery. That Shiga toxin does induce signaling events is illustrated by its activation of the *src*-like kinases *yes* and *lyn*.[27,28] How subsequently adaptors and clathrin triskelions would be recruited to the membrane, or whether the toxin containing rafts would enter preformed pits, is unclear.

Entry via Non-Clathrin Dependent Routes

Of the 5 toxins presented here, most of them can enter the cell by clathrin dependent as well as clathrin independent mechanisms, but only VacA was found to enter exclusively via a clathrin-independent pathway,[18] but the exact pathway has not been further documented other than that it is lipid raft dependent.[13,18,29,30] In contrast, the alternative entry routes were extensively studied for CT.[1,22,23,31,32-34] The common denominator of the clathrin-independent CT entry pathways is their dependence on cellular cholesterol.[12,22,34] Uptake through caveolae has been frequently proposed[1,31,33] however several recent studies have concluded that it is a relatively minor pathway.[22,23,32,34] Using mouse embryonic fibroblasts form caveolin-1 knock out mice, Parton and colleagues[34] have recently shown that CT preferentially enter via two pathways besides the caveolar pathway : the clathrin-coated pit pathway, as mentioned above, and a nonclathrin noncaveolar cholesterol dependent pathway, that is also used by glycosylphosphatidyl inositol (GPI) anchored proteins.[35] Massol et al recently found that although endocytosis was not longer detected, cytotoxicity of CT was retained in cells cotransfected with dominant negative mutants of dynamin (involved in both the clathrin and caveolar pathways) and Arf6 suggesting the existence of a pathway independent of clathrin, caveolin and Arf6.[25] Depending on the cell types, ST was also found to enter cells via a clathrin and dynamin independent pathway,[36,37] but this route was not studied in detail.

Early Endosomes: Toxin Translocation Site or Transit Area

Despite the diversity of the entry requirements and routes, all five toxins reach the early endosomes. The route they have followed might however determine their precise localization within the early endosomes, since this compartment is composed of a mosaic of membrane domains,[38,39] In addition the compartment is polymorphic with tubular and cisternal regions, the later being multivesicular.

For DT, the early endosome is the final destination.[40,41] The acidic milieu of the endosome triggers a conformational change in the B subunit of the toxin that inserts into the endosomal membrane and allows the translocation of the A subunit into the cytoplasm. At that stage, a proteolytic cleavage has occurred between the A and B subunits, which however remain attached via a disulfide bound that becomes reduced by cytosolic factors, possibly thioredoxin reductase.[42] Translocation occurs in an at least partially unfolded state and refolding on the cytoplasmic side is promoted by the cytosolic chaperone HSP90, together with other yet to be identified cytosolic factors.[42] Once in the cytoplasm, the A subunit ADP-ribosylates Elongation Factor 2 (EF2), leading to the inhibition of protein synthesis followed by cell death.[3]

CT and ST are only in transit in early endosomes from where they are directly routed, i.e., without going through later endocytic compartments, to the Trans-Golgi Network (TGN). The association of the gangliosides, GM1 for CT[43] and Gb3 for ST,[44] with detergent resistant membranes, and presumably with lipid rafts, was found to be essential for transport to the TGN. More over transport of ST to the TGN requires clathrin, dynamin, epsinR, and possibly Rab11.[36,37,45] Also two different SNARE complexes, involving syntaxins 5 and 16, were found to be important.[46] This direct retrograde pathway, described for first time for ST, appears to be used by a growing number of Golgi proteins that cycle between the Golgi, the plasma membrane and early endosomes.[47] CT and ST are subsequently transported to the Golgi (Fig. 1) and the endoplasmic reticulum from where they undergo retrograde translocation into the cytoplasm, hijacking the cellular machinery that enables misfolded proteins to cross the ER membrane to reach the cytosol where proteasomal degradation can take place (for review see ref. 48). Once in the cytoplasm, the CT A subunit ADP-ribosylates G proteins leading to the increase of cAMP and subsequent chloride secretion, where as the ST A subunit cleaves a single Adenine base from the 28S ribosomal RNA thus inhibiting protein synthesis.

VacA and anthrax toxin are also in transit in early endosomes on their way to late endosomes. Anthrax toxin was found to require sorting into the cisternal regions of the early endosomes as opposed to the tubular regions involved in recycling.[41] More specifically the toxin is sorted, by yet unknown mechanisms, into nascent intraluminal vesicles of the early endosomes. The biogenesis of intraluminal vesicles is described in Chapter 8 and involves the ubiquination of cargo molecules and the interaction with the three ESCRT (Endosomal sorting complex required for transport) complexes. Once sorted into these vesicles, the heptameric anthrax B subunit inserts into the membrane,[41] in a manner that is dependent on the acidic endosomal pH, and mediates the translocation of the A subunits, of which anthrax toxin has two: lethal factor LF, a metalloprotease that cleaves MAP kinase kinases, and edema factor EF, a calmodulin dependent adenylate cyclase. Since B subunit channel formation occurs in the membrane of intraluminal vesicles, the enzymatic subunits end up in the lumen of these vesicles upon membrane translocation[41] (Fig. 1). Upon budding of multivesicular bodies, or endosomal carrier vesicles (see Chapters 1, 2 and 8), the encapsulated enzymatic toxin subunits are thus withdrawn for the early endosome and transported to late endosomes. As for early to late endosomal transport in general, transport of anthrax toxin A subunits was dependent on the integrity of microtubules.

Late Endosomes: Who Can Make It That Far and What For?

Whereas most, if not all, AB type toxins interact at some point with early endosomes, very few reach later stages of the endocytic pathway. In fact, only two toxins have been described to do so: vacA and anthrax toxin.

The most striking feature of vacA, which led to its name, is that it triggers vacuolation of cells. The compartment undergoing vacuolation was subsequently identified as the late endosome based on its acidic lumen, the presence of lamp1 and rab7[49-51] but not of early endosomal markers. The current model to explain the mechanism by which VacA induces vacuole formation is by forming anion-selective channels leading to swelling of endosomal compartments.[52-54] What remains unclear is why channel formation would preferentially occur in late endosomes, especially since vacA was also shown to form channels in the plasma

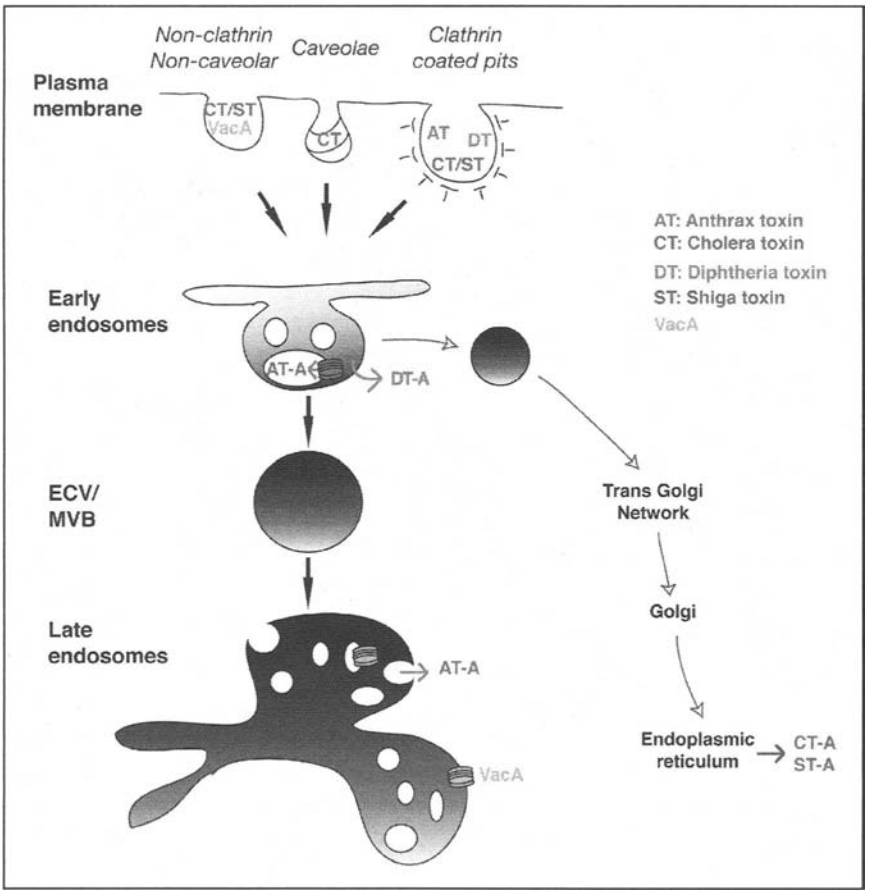

Figure 1. Schematic representation of the intracellular routes followed by anthrax toxin (AT), cholera toxin (CT), Diphtheria toxin (DT), Shiga toxin (ST) and VacA. Toxins can be internalized by a variety of pathways including the clathrin dependent, the caveolar and the clathrin and caveolin independent routes. They are subsequently transported to early endosomes which is either the site from where translocation into the cytoplasm occurs (as for DT) due to membrane insertion of the B subunit at the acidic endosomal pH. Other toxins such as CT and ST are sorted, using protein and lipid based mechanisms, to the trans Golgi Network for subsequent transport to the endoplasmic reticulum where retrotranslocation of the enzymatic A subunits occurs. Yet other toxins such as vacA and anthrax toxin are transported from early endosomes to late endosomes. In this late compartment, vacA forms transmembrane channels thus leading to alterations of the compartment. In contrast anthrax toxin does not alter the compartment but uses it as a entry site to the cytoplasm.

membrane, and in mitochondria.[4] This raises the possibility that channel formation may also occur in early endosomes but does not trigger swelling of the compartment. Note that VacA induced vacuolation only occurs in the presence of weak bases such as ammonium chloride. In the absence of weak bases, despite the absence of vacuolation, alterations of late endosomes where however observed such as inhibition in EGF degradation and procathepsin D maturation[55] or clustering of late endosomes.[51]

In contrast to vacA, anthrax toxin does not alter late endosomes but uses them as a portal of entry into the cytoplasm. As mentioned above, the enzymatic subunits of the anthrax toxin, the

lethal factor LF and the edema factor EF, are translocated, at the early endosomal level across the channel formed by the protective antigen from the lumen of the endosomes to the lumen of intraluminal vesicles (Fig. 1). These are subsequently incorporated into endosomal carrier vesicles (ECV), i.e., the transport intermediate that mediate trafficking between early and late endosomes, which are transported along microtubules to the perinuclear regions of the cells where fusion with late endosomes can occur. The requirement for transport of EF and LF to late endosomes is supported by the fact that depolymerization of microtubules using nocodazole or expression of dominant negative rab 7 inhibit the action of LF.[41] Once arrived in late endosomes, EF and LF are thought to await back fusion events between the intraluminal vesicles and the limiting membranes, an event that would lead to release into the cytoplasm. Back fusion between intraluminal vesicles and limiting membrane is a very poorly characterized phenomenon. The occurrence of such events is however supported by the observations that certain proteins found on intraluminal vesicles are merely there on transit. These include the mannose-6-phosphate receptor that is subsequently routed to the Golgi, to capture newly synthesized lysosomal enzymes, as well as major histocompatibility complex II that must reach the plasma membrane once peptide loading has successfully taken place.[56,57] Although the requirement for back fusion has not yet been shown for the anthrax toxin, due to the lack of knowledge concerning this mechanism, the importance of dynamics of intraluminal membranes is supported by the inhibitory effect of antibodies directed towards a lipid almost exclusively found on internal membranes, namely lysobisphosphatidic acid (see Chapter 2). Down regulation of the protein Alix, the mammalian homologue of the yeast class E vacuolar protein sorting vps31, involved in multivesicular body sorting and biogenesis[58,59] was also found to inhibit delivery of LF to the cytoplasm.[41] The benefit of delivering the enzymatic subunits of anthrax toxin to the cytosol from late rather than early endosomes has not yet been demonstrated. It is however attractive to believe that late endosomes act as a delivery platform of these subunits to the vicinity of their early targets.

Concluding Remarks

Bacterial toxins are the fruit of a long-term coevolution between pathogenic bacteria and their host. Trial and error, or attack and counter-attack, have shaped them into very sophisticated weapons that utilize host cell machineries in their greatest intimacy. More over different toxins have developed different strategies in particular to reach the cytoplasmic milieu. Advances in cell biology have considerably helped to better understand the modes of actions of toxins, but importantly the studies of toxins have brought to light unknown or poorly understood intracellular trafficking routes. There is no doubt that these undesired foreigners still have a lot to tell us and will contribute to further characterization of the mechanisms that govern endocytic trafficking.

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