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Not just a sink: endosomes in control of signal transduction

Marta Miaczynska[1], Lucas Pelkmans and Marino Zerial[2]

Recent studies indicate that endocytic organelles can play a more active role in signal propagation and amplification than was recognised before. By deciphering the interplay between endocytosis and signalling, we will be able to gain a more sophisticated level of understanding of signal transduction mechanisms.

Addresses
Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany
[1]e-mail: miaczynska@mpi-cbg.de
[2]e-mail: zerial@mpi-cbg.de

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Abbreviations

EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
GPCR	G-protein-coupled receptor
MAPK	mitogen-activated protein kinase
NGF	nerve growth factor
PDGF	platelet-derived growth factor
PI(3)P	phosphatidylinositol 3-phosphate
SARA	Smad anchor for receptor activation
TGF-β	transforming growth factor β
TGF-βR	transforming growth factor β receptor

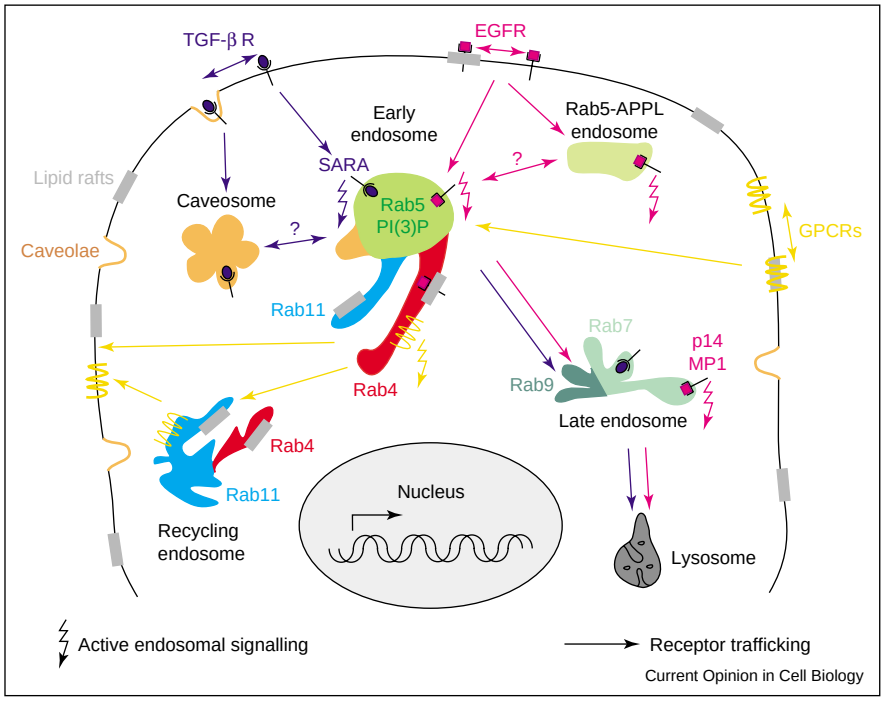
Introduction

Cells possess an elaborate and highly structured signal transduction machinery to translate responses to external stimuli into programmed changes in gene expression. The classical model of signalling involves membrane-spanning receptors that, after binding an extracellular ligand (e.g. growth factors or hormones) at the cell surface, activate secondary messengers in the cytosol, enabling the spread of the signal into the nucleus. Endocytosis has long been recognised as a means to terminate signalling via degradation of activated receptor complexes after their internalisation from the cell surface. Because endocytosis has been considered to be a mere 'sink' of signalling complexes, attention has mainly been focused on the identification of components of the signal transduction cascade and their interactions. However, it has become clear that the output of a signalling process depends not only on activation of a particular set of signalling molecules but also on where and for how long the signal is emitted. Exciting new findings suggest that the signalling machinery can achieve a high order of regulation by exploiting the compartmentalisation and functional specialisation of the endocytic pathway, going beyond its conventional role in cargo degradation. Endosomes appear to be ideally suited for such regulation as they are organised as a network of physically and biochemically distinct membranous domains, interconnected by a tightly controlled transport system (Figure 1) [1–4]. Endosomal proteins are not randomly distributed but localized to specific domains that are arranged in a mosaic fashion. Recent work in a variety of cellular and developmental systems supports the proposal that endocytic organelles can play a direct role in signal propagation and amplification (see for example review by Dudu, Pantazis and Gonzalez-Gaitan in this issue). In this review we focus on the role of endocytic organelles as intracellular signalling stations and the emerging concepts explaining how spatial and temporal compartmentalisation of signal transduction in the endocytic pathway may contribute to signalling specificity and regulation.

The concept of signalling from endosomes

The prevailing view that signalling occurs on the plasma membrane only was challenged in the early nineties by Bergeron, Posner and colleagues, who observed that shortly after ligand addition the majority of activated epidermal growth factor receptors (EGFRs) and their downstream signalling factors such as Shc, Grb2 and mSOS were found not on the plasma membrane but on early endosomes [5], suggesting that EGFR signalling continues from this compartment [6]. Subsequent demonstration that nerve growth factor (NGF) was bound to its activated receptor TrkA and phospholipase C-γ1 (PLC-γ1) in endocytic organelles [7] led to the 'signalling endosomes' hypothesis. However, the presence of signalling complexes on endosomes could arguably reflect a transport intermediate on the way to lysosomal degradation. For this reason, and because signalling events on the plasma membrane always precede those on endosomes, the participation of endocytosis in signalling was largely neglected. However, Schmid and colleagues demonstrated that prolonged residence of activated EGFR on the plasma membrane led, as a result of impaired clathrin-mediated endocytosis via expression of Dynamin^{K44A}, to reduced activity of some downstream signalling components, such as mitogen-activated protein kinases (MAPKs) ERK1/2 or the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) [8]. A similar reduction in MAPK

Figure 1



Signalling in endocytic compartments. Targeting of ligand–receptor complexes into different endocytic domains and compartments is proposed to directly regulate signal transduction through localized assembly of specific effector complexes. On the plasma membrane, receptors such as EGFR (magenta), TGF-β R (black) or GPCR (yellow) are present in non-raft, raft or caveolar domains. Upon ligand binding they undergo internalisation into various types of endocytic organelles, early endosomes, caveosomes or APPL-positive endosomes. Since the endocytic compartments are composed of distinct domains, including Rab-domains, rafts and caveolar domains (marked with different colours), the signalling output will depend on the local membrane environment. Sorting determinants on ligand–receptor complexes direct them to degradation (EGFR, TGF-β R) or along the recycling pathway (GPCRs). GPCR signalling continues post-internalisation through association with β-arrestins. EGFR signalling occurs throughout the endocytic journey, on early endosomes (via mSOS-Shc-Eps8), APPL endosomes (via APPL) and late endosomes (via p14-MP1). TGF-β R signals via SARA and Smad2 on early endosomes but remains inactive in caveolar domains or caveosomes. Importantly, TGF-β signalling through the interaction with SARA is restricted to a Rab5-domain on early endosomes enriched in PI(3)P. Other Rab-domains (Rab4 and Rab11 domains) in early and recycling endosomes may exclude SARA, and thus may not be signalling-competent because of the lack of PI(3)P. Magenta, black and yellow arrows represent the trafficking of EGFRs, TGF-β Rs or GPCRs, respectively.

activation was observed upon inhibiting endocytosis of β_2-adrenergic receptor (β_2-AR), a member of the G-protein-coupled receptor (GPCRs) family [9]. These data indicated that some downstream cascades are preferentially activated after internalization of receptor-ligand complexes into endosomal compartments. Similarly, EGFR was shown to interact on endosomes with Grb2, an initiator of Ras and MAPK signaling, demonstrating that signal transduction can in principle continue after endocytosis [10]. Recent computational modeling of the dynamics of EGF signal transduction via MAPK cascade further predicts that at low (i.e. physiological) ligand concentrations internalised EGFR continues to signal and contributes significantly to the overall cell response [11].

Additional biochemical evidence documenting the role of endosomes in EGF and platelet-derived growth factor (PDGF) signal propagation has been obtained more recently by using specific reversible inhibitors of EGFR and PDGFR tyrosine kinases to cause internalisation of ligands bound to inactive non-phosphorylated receptors [12•,13,14]. These complexes were then specifically activated in endosomes upon inhibitor wash-out and were able to recruit signalling molecules and to elicit biological responses. Interestingly, studies describing selective isolation of internalised EGFR by use of reversibly biotinylated antibodies [15] revealed that although both plasma-membrane and endosomal pools of EGFR remained active, some downstream signalling molecules were preferentially associated with one of these pools, for example Grb2 with the surface and Eps8 with the endosomal EGFR pool. These data point again to qualitative differences in signalling emitted from these two locations. But the questions raised by these observations are why and by which mechanisms should endosomes be involved in the propagation of signals throughout the cytoplasm to the nucleus.

Why signalling via endosomes?

Temporal regulation

One obvious role for endocytosis in signalling is to provide temporal regulation, as the duration of signalling is an important parameter determining the biological output. The lifetime of signalling complexes assembled upon activation of plasma membrane receptors depends on several parameters, including the kinetics of receptor internalisation and their subsequent destination once delivered into endosomes. The duration of the signalling process depends on the proportion of receptors undergoing degradation compared to those recycling to the plasma membrane [16,17]. Even within one receptor family these parameters can differ significantly. For example, upon binding EGF, EGFR (ErbB1) is rapidly internalised into endosomes where it remains active for several minutes before sorting to lysosomes for degradation, with a significant proportion (25–30%) being recycled [17,18]. In contrast to ErbB1, which appears to signal from endosomes for most of its lifetime in the active state, other family members (ErbB2, -3 and -4) are endocytosed very inefficiently upon EGF treatment, remaining active for longer periods of time on the plasma membrane. Dimerisation between various ErbB molecules modifies their trafficking properties further, affecting the duration of signalling at the plasma membrane versus endosomes. Different transport kinetics could thus account for the observed differences in signalling response [17,19–21]. In addition, various ligands for EGFR/ErbB receptors, such as EGF or transforming growth factor-α (TGF-α), exhibit differential sensitivity to acidic endosomal pH, which affects their dissociation from the receptors and, as a consequence, their intracellular trafficking [22].

As another example, the kinetics of agonist-stimulated internalisation and transport along the endocytic pathway differs between different members of GPCR family. In general, agonist treatment causes GPCR phosphorylation and binding to β-arrestins, resulting in receptor desensitisation and internalisation [23,24]. β-arrestins act as a scaffold for intracellular assembly of signalling complexes, including components of MAPK and c-Jun amino-terminal kinase 3 (JNK3) pathways [25]. Dissociation of β-arrestins allows GPCR recycling via Rab4- and Rab11-dependent mechanisms (Figure 1) or targeting to lysosomes [26•]. The affinity of β-arrestins for a particular GPCR regulates what route the receptor takes and its lifetime in endosomes [27,28], providing a possible explanation of why various GPCRs depend to a different degree on endocytosis in eliciting their intracellular responses.

Targeting signalling complexes to their site of action

Communication between endocytic organelles requires actin- and microtubule-dependent motility [1,29,30]. In response to growth factors, endosomes may modify their motility properties through local changes in the cytoskeleton, for example via endosomal Rho proteins, their effectors and *Src*-kinases [31]. Movement of early endosomes on microtubules relies on Rab5 and PI3-K [1]. Given their ability to stimulate Rab5 and PI3-K, signalling molecules may modulate the recruitment or activity of microtubule motors on endosomes, thereby regulating their motility and intracellular distribution. The movement of endosomes can direct activated signalling molecules to their target site [32]. For example, protein kinases and phosphatases exert opposite effects in the signalling cascade and mathematical modelling predicts a dramatic decline in the active phosphorylated species with distance from the plasma membrane to the nucleus [33,34••]. This argues for the necessity of a membrane vehicle, i.e. endosomes, to actively transport signalling complexes to their site of action before they become inactivated. Particularly illustrative is the case of neurons, where signals from axon terminals must travel long distances to reach the cell body. Here, simple diffusion through the cytoplasm is insufficient to transmit the signal. Instead, signalling is facilitated through microtubule-mediated retrograde transport of signalling complexes in endocytic vesicles, such as those containing NGF bound to its receptor TrkA (reviewed in [35]). These vesicles, recently isolated from dorsal root ganglion neurons [36••], correspond to early endosomes, as they contain Rab5 and its effector EEA1 [1], and carry signalling-competent complexes (including phospho-ERK1/2, phospho-MEK1, phospho-MAPK p38, B-Raf, Gab2 and Rap1). Furthermore, NGF–TrkA internalisation and retrograde transport were shown to be necessary for neuronal survival, probably because TrkA kinase activity can be maintained in endosomes as they travel along the axon [37••]. Interestingly, such active transport enables the generation of different signalling outputs depending on the location; for example, localised signals are produced at the site of stimulation in the nerve terminal, whereas generalized cellular responses are evoked in the cell body [38].

Despite the retrograde vesicular transport of NGF–TrkA, alternative models for NGF signal propagation have been put forward (reviewed in [35,39,40]). One proposal, termed the 'domino' or 'wave propagation' model, envisages a rapid lateral propagation of TrkA phosphorylation along the axonal plasma membrane, initiated by NGF binding in the terminal but spreading in a ligand-independent fashion [41]. A similar wave of lateral receptor phosphorylation was visualised by fluorescence imaging following focal stimulation of EGFR in non-neuronal cells [42]. Another, 'retrograde effector' model proposes that signalling molecules activated by NGF–TrkA reach the cell body independently of the ligand–receptor complexes. Support for non-endosomal NGF signalling comes from studies where the axon terminal is stimulated focally with NGF-coated beads, which cannot be

internalised but are capable of eliciting neuron survival [43]. Although NGF beads caused activation of TrkA and Akt, MAPK phosphorylation was nevertheless impaired under these conditions, demonstrating again a role for endosomal targeting in the selective activation of signalling pathways. Cumulatively, the data on NGF action strongly argue that signalling from endosomes is of functional importance, although other mechanisms may act in parallel to provide robustness to the system as well as fine-tuning. This paradigm is expected to operate also in non-neuronal cells in response to factors other than NGF [34••]. The example of neurons indicates that endosomes, with their cytoskeleton-dependent motility, are ideally suited for a precise, directional and controllable delivery of signalling complexes into specific cellular locations, a function that cannot be reproducibly and accurately achieved by signalling wave propagation or diffusion-based mechanisms.

Spatial regulation

Given that several cytoplasmic signalling molecules are shared between different pathways, how is unspecific cross-talk between these pathways prevented? Unwanted interactions may be avoided by restricting signalling cascades in space. Besides acting as transport carriers over long distances, endosomes may provide two additional interconnected levels of control: selective targeting of signalling molecules to specific organelles and their segregation to subdomains within a given organelle.

Well-known membrane subdomains involved in signalling are immunological synapses in lymphocytes [44] and focal adhesion sites in epithelial cells [45]. Another, more general type of subdomain present in most cellular membranes is based on the biophysical properties of particular lipid species. In the extracellular leaflet of membrane bilayers, saturated glycosphingolipids may come together to form liquid-ordered domains intercalated by cholesterol, called lipid rafts [46]. Similar microdomains are suggested to exist also on the cytosolic leaflet and to be linked to the outer leaflet rafts [47]. Interestingly, several signalling molecules, including receptor tyrosine kinases, GPI-anchored signalling molecules, certain Ras-family members and lipid-modified molecules, like most *Src*-family kinases, display high affinity for lipid rafts. By regulating the partitioning of signalling components into lipid rafts, signalling cascades could either be initiated or be terminated [48]. A specialised form of lipid rafts present in most cell types, called caveolae, are small, flask-shaped invaginations stabilised by oligomers of caveolin-1, the principal structural component of caveolae [49]. Besides its structural role, caveolin-1 is thought to recruit several signalling proteins through its scaffolding domain [50]. Although the precise mechanism remains unclear, it is nevertheless possible that signalling cascades might be regulated via recruitment of signalling molecules into caveolae. For example, caveolae appear to negatively regulate the signalling functions of EGFR, PDGFR, GPCRs and nitric oxide synthase by preventing their interactions with downstream effectors [51].

Because lipid rafts and caveolin-1-positive structures travel through endocytic and exocytic compartments, the partitioning of signalling molecules into these microdomains may be a general mechanism operating on most intracellular organelles. In fact, a compelling case for the role of caveolae in signalling is related to their ability to internalise [52•]. When caveolae internalise, they can be targeted to intracellular organelles enriched in caveolin-1, called caveosomes. Caveosomes provide both a luminal milieu and a membrane environment that is distinct from early or late endosomes [53]. Interestingly, it appears that selective targeting to either caveosomes or early endosomes specifically modulates the signalling by TGF-β receptors [54•]. In the TGF-β signalling network, SARA (Smad anchor for receptor activation) is specifically recruited to a Rab5-domain on early endosomes enriched in phosphatidylinositol 3-phosphate (PI(3)P) [55,56] (Figure 1). Interestingly, SARA appears to play a dual role on endosomes, regulating both cargo trafficking through the Rab5 compartment and receptor signalling from this domain [57,58]. Engagement of TGF-β receptor with SARA is necessary to phosphorylate Smad2 and to achieve subsequent propagation of the signal, explaining why TGF-β receptor internalisation into early endosomes is necessary for proper signalling. In caveosomes, however, TGF-β receptors do not encounter SARA and remain inactive. An intriguing observation is that expression of constitutively inactive Rab5 (Rab5S34N) stimulates TGF-β signalling, while expression of constitutively active Rab5 (Rab5Q79L) has no effect [57]. One possibility is that Rab5 inhibition may modify the transport of TGF-β receptors between caveosomes and early endosomes, thus underscoring the influence of trafficking on signalling.

Scaffold factors can play an important role in signalling by regulating the trafficking of signalling receptors and/or the activity of downstream signalling components, such as MAPK [25,32]. Interestingly, these molecules can also specifically localise signalling complexes to endocytic compartments. Huber and colleagues identified a late endosomal protein p14, which was indispensable for efficient EGF signalling in the ERK cascade [59••]. Localisation of the MP1–MAPK scaffold to late endosomes via p14 (Figure 1) was required for full ERK1/2 activation in a later phase of signalling after initial activation at the plasma membrane. Interestingly, mistargeting of p14-MP1 to the plasma membrane could not compensate for lack of endosomal signalling, arguing that late endosomes provide a unique platform for signal propagation.

During their endocytic itinerary, receptors destined for degradation, such as EGFR, are sequestered into internal

vesicles that form from the limiting membranes of the endosomal vacuoles, a process that starts at the early endosome and leads to formation of multivesicular bodies and late endosomes [60]. As receptors within the intra-endosomal vesicles are considered inactive, signalling must occur within the time span of transport between the plasma membrane and incorporation into intra-lumenal domains. It is important to consider, however, that intra-lumenal sorting of proteins away from the cytoplasm does not always result in degradation, as the internal vesicles containing MHC class II molecules can be used as storage containers that fuse back with the limiting membrane [61]. By modulating a balance between active signalling complexes on the limiting outer membrane versus the sequestered inactive molecules in the internal vesicles, late endosomes may provide yet another mechanism to regulate the signal quality and duration.

Are there specialised endosomes primarily devoted to signalling? An endocytic structure, seemingly distinct from canonical early endosomes and bearing the small GTPase Rab5 together with its two effectors APPL1 and APPL2, was recently identified as an intermediate in signalling between the plasma membrane and the nucleus [62••]. APPL proteins are essential for cell proliferation, capable of nucleocytoplasmic shuttling in response to EGF or oxidative stress and associate directly with nuclear proteins (the NuRD/MeCP1 complex). Other endocytic proteins, such as eps15, epsin1, CALM (clathrin assembly lymphoid myeloid leukemia) and α-adaptin were previously shown to undergo translocation from the plasma membrane to the nucleus [63]. In contrast, the localisation of APPL proteins to a hitherto undescribed population of endocytic membranes, together with their essential role in mitogenesis, suggest that certain responses within EGF signal transduction pathway may be triggered from a subset of specialised endosomes.

Conclusions and future prospects

The data available so far demonstrate that signal transduction occurring from endosomes has functional importance, although different modes of signal propagation may co-exist for a given stimulus in the same cell and different signalling systems may depend on endosomes to various degrees [23,34••]. By sorting and trafficking ligand–receptor complexes within defined timeframes, endosomes provide temporal control of signal transduction. Activated plasma membrane receptors can be internalised via several routes (e.g. clathrin-mediated, caveolin-dependent, clathrin- and caveolin-independent, macropinocytosis) into different types of endosomes (e.g. EEA1- or APPL-positive ones). By providing a platform for compartment-specific molecular interactions leading to the assembly of unique signalling complexes, endosomes also add a level of spatial control to signal propagation events. Targeting to different endosomes or even distinct membrane subdomains within the same endosome can determine specific cellular responses. From this point of view, metabolic networks provide a good example of how compartmentalisation is essential for the correct flow of enzymatic reactions [64]. Moreover, the same membrane–cytoskeleton interactions that regulate the position of endosomes within the cell can be exploited for the generation of localized signal transduction responses, thus providing an advantage over diffusion- or lateral-wave-propagation-based mechanisms.

Although in the past few years the interface between endocytosis and signalling has been actively explored, we still do not know how preferential activation of particular signalling cascades on endosomes translates into specific biological responses. Determining the subcellular localisation of signalling components and their interactions with respect to the subdomain organisation of endosomes is bound to reveal new insights into how signalling networks operate. It remains to be clarified whether and how sorting of receptor complexes into intra-endosomal vesicles during the formation of multivesicular bodies correlates with the termination of endosomal signalling. Furthermore, it is clear that responses generated shortly after growth factor exposure are not limited only to the plasma membrane and endosomes. Intriguingly, upon receptor tyrosine kinase endocytosis, phosphatidylinositol-3,4,5-triphosphate is produced on endoplasmic reticulum (ER) and Golgi membranes [65]. Similarly, the ER appears to be a site of EGFR and PDGFR dephosphorylation following their internalisation [66]. In addition to its established role on the plasma membrane and more recently recognised function in endosomes ([67,68]; reviewed in [69]), Ras is localized to the ER and Golgi complex where it can be activated by specific exchange factors [70]. Dynamic interactions between the ER, the plasma membrane and the endosomal system contribute to phagosome formation and antigen cross-presentation in macrophages and dendritic cells [71–73], thus underscoring the plasticity of these compartments under different physiological conditions. These observations raise the intriguing question of how the extensive communication between endosomes and the organelles of the biosynthetic pathway contributes to the repertoire of regulatory mechanisms of the cellular signalling machinery.

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