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SIGNALING ENDOSOMES: SEEING IS BELIEVING

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Summary

Signaling compartmentalization provides a highly refined mechanism to specify context-dependent cellular responses. Endosomes are an intracellular membrane-bound compartment that mediates the transport of receptor-bound signaling complexes. Owing to the development of high-resolution microscopy-based imaging techniques it has been possible to demonstrate that endosomes are actively engaged in signal reception and emission. Such observations paved the way to functional studies ascribing indispensable roles for endosomes in orchestrating signals that regulate processes such as cell migration and invasion, asymmetric cell division and differentiation, or intercellular communication.

Introduction

As the barrier between the cell exterior and interior, the plasma membrane has been long viewed as the principal site for the transmission of extracellular signals. This view has been firmly supported by multiple studies demonstrating that signaling complexes consisting of molecules that reside on the external and internal surface of the plasma membrane are critical for the relay of extracellular signals to designated cytoplasmic effectors. In the mid-nineties, biochemical fractionation studies have identified activated growth factor receptors and their associated signaling molecules in subcellular fractions containing endosomes [1,2]. These findings raised the intriguing possibility that signaling territories might extend beyond the plasma membrane and that endosomes may act as intracellular platforms for compartmentalized signaling. Implicit in this idea is the prediction that by ferrying active signaling cargo from the cell membrane to the cytoplasm, endosomes may serve as a vehicle for directional and controllable delivery of signaling complexes into specific locations, acting more specifically than diffusion-based signal propagation. Indeed, mathematical and computational approaches to model kinetic parameters of signal transduction from the plasma membrane to the cell nucleus have postulated that active endosomal transport of phosphorylated MAP kinases would be necessary to prevent their rapid dephosphorylation by cytoplasmic phosphatases [3]. Furthermore, in neuronal cells the cytoskeleton-based motility of endosomes has been implicated in the directional transport of signaling molecules to distinct sites [4–6]. These early observations have been corroborated by many subsequent studies documenting the participation of endosomes in various aspects of intracellular signaling.

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Key to the advancement of research on the involvement of endosomes in specifying signaling output has been the development of imaging techniques that permit the visualization of endosome-associated signaling events at a high spatio-temporal resolution. This necessitated the establishment of tools to track ligands, their receptors and/or key signaling molecules by fluorescence microscopy in fixed and living cells, tissues or even organisms (Figure 1). Here we will focus on the application of such novel microscopy-based methods and tools to document the roles of endosomes as signaling organelles. Due to space constraints we will highlight a few examples in which high-end visualization methods have proven critical to the discovery process. The more general topic of endosomal signaling has received comprehensive coverage in recent reviews [7,8].

Tools to detect signaling molecules on endosomes

Labeled ligands/receptors

Epidermal growth factor (EGF) signaling has been among the first systems to be characterized with respect to its connection with endocytic trafficking owing to the fact that fluorescently tagged versions of EGF became commercially available early on [9,10]. In the past decade, the use of tagged forms of ligands to track their intracellular trafficking has been extended to additional growth factors, cytokines or and hormones. Most of such ligands are labeled with small-molecule fluorophores (e.g. FITC, rhodamine, Alexa Fluor™ dyes), conjugated either directly or indirectly by employing biotinylated ligands and fluorophores coupled to streptavidin. The latter method has also been used for the attachment of streptavidin-covered semiconductor quantum dots (QDs) to biotinylated ligands such as EGF or nerve growth factor (NGF) [11–13], although the direct conjugation of EGF to QDs has been achieved as well [14]. Antibodies recognizing extracellular parts of the receptors and undergoing internalization in complex with them have been also employed to monitor the intracellular trafficking of receptors [15,16]. Additionally, genetically engineered constructs of tagged receptors and tag-specific antibodies have been used for the same purpose [17,18]. In contrast to the visualization of all receptors in the cell, application of fluorescent ligands or antibodies internalized with the receptors results in the specific detection of those receptors which underwent endocytosis. Disadvantageous in this approach can be high, non-physiological concentrations of the labeled ligand which are often required to obtain sufficiently bright signals for imaging. Despite certain limitations, various visualization tools enable a rather precise delineation of the endocytic routing of a particular ligand/receptor (e.g. clathrin- or non-clathrin-mediated internalization, sorting for degradation or recycling). These approaches have been successfully used and paved the way to functional studies in which pharmacological or RNAi-mediated “re-routing” of a ligand/receptor complex was shown to have an impact on particular signaling events [19–23].

Fluorescence resonance energy transfer (FRET)

Amenable to use in living cells, FRET has been the principle method by which the interactions of signaling molecules on endosomes can be assessed in real-time. In this approach, two matching fluorophores (donor and acceptor) are attached to two molecules of interest. If such molecules interact, close proximity between the fluorophores leads to non-radiative energy transfer from the donor to the acceptor, thus resulting in higher (“sensitized”) acceptor emission and in quenched donor emission which can be measured upon excitation of the donor [24]. A notable early example of FRET application to study endosomal signaling includes the visualization of the interaction between the EGF receptor (EGFR) and the signaling adaptor molecule Grb2 in membrane ruffles and on endosomes [25]. FRET technology has been further used to monitor the activation states of molecules. In such cases, one of the interaction partners is a peptide or a protein domain exhibiting activation state-specific binding to the second partner. For example, Ras-binding domain of

Raf (Raf-RBD) interacting specifically with Ras-GTP has been used as a sensor of Ras activity in a so-called Raichu probe, consisting of YFP, Ras, Raf-RBD and CFP with the farnesylation signal, all these parts fused as one molecule [26] (Figure 2a). Upon activation of Ras, its intramolecular binding to Raf-RBD brings CFP and YFP in a close proximity resulting in FRET. Such Raichu-KRas probe has been recently used to document the specific activation of K-Ras but not H-Ras or N-Ras on late endosomes thus identifying a mechanism for intracellular signal propagation [27]. A recent application of another FRET sensor, termed Epac1-camps, has allowed a detection of cAMP responses elicited from endosomes upon the action of thyroid-stimulating hormone (TSH) [28] (see also below for more details). The Epac1-camps sensor consists of a CFP-YFP pair containing a cAMP-binding domain of Epac1 fused in between them [29]. Increased cAMP levels induce a conformational switch of this domain which leads to increased distance of the fluorophores, thus reducing their FRET.

Fluorescence lifetime imaging microscopy (FLIM)

Even more precise measurements of protein-protein interactions and their localization in intact living or fixed cells can be achieved by a combination of FRET with FLIM using confocal or multiphoton illumination. FLIM enables measurements of fluorescence lifetime values, rather than fluorescence intensities, within a microscopical image. Fluorescence lifetime is a characteristic feature of each fluorophore and does not depend on its local concentration in the sample, the intensity of excitation or the spectral bleed-through between FRET donor and acceptor [30]. In contrast, all these factors may present certain limitations in the steady-state FRET technique measuring fluorescence intensities, as described above. Despite these advantages, FRET-FLIM remains technically very demanding and has not been applied as widely as FRET. Still, FRET-FLIM has been used to detect endosomal formation of signaling complexes between the chemokine receptor CXCR4 and protein kinase C α [31] or between fibroblast growth factor receptor 2 (FGFR2) and its major signaling effector FGFR substrate 2 (FRS2) [32].

Photoactivatable fluorescent proteins (PA-FPs)

are being increasingly applied to detect specific subpopulations of signaling molecules on individual endosomes. A key feature of PA-FPs is their ability to change spectral properties upon specific light-induced activation. Such changes include conversion to a fluorescent state or switching between two fluorescent colors [33]. Local activation of PA-FPs in a specific region of a cell allows visualization of only a particular subset of molecules labeled by PA-FPs, despite their presence throughout the cell. This provides an important advantage for reliable tracking of individual endosomes which presents a serious challenge when all endosomal structures are labeled, due to their high number, density and dynamics. Recently, real-time imaging of photoactivatable GFP- $\alpha 5$ integrin during invasive cell migration in three-dimensional matrices has been used to demonstrate its continuous recycling via Rab25-positive endosomes within the tip regions of extending pseudopods [34]. Similarly, the dynamics of individual endosomal vesicles carrying Rac GTPase was recorded using fusion protein with photoactivatable GFP [35] (see also below). Photoactivatable GFP- β -catenin has been used to document the accumulation of β -catenin/E-cadherin complexes in perinuclear recycling endosomes potentially as the means to facilitate β -catenin relocation into the cell nucleus in response to Wnt signaling [36].

Functional studies of signaling endosomes employing high-resolution microscopy

While the presence of active signaling complexes on endosomes is currently well documented, establishing their functional significance has proven to be a difficult task.

Particularly challenging has been the distinction between the signaling events elicited initially on the plasma membrane from those originating from the endocytic organelles. In the following section, we will focus on selected recent studies in which the use of high-end microscopy methods afforded the discoveries of various functions of endosomes in specific signaling systems.

Endosomes in polarized cell migration

Although continuous membrane flow, including endocytosis and recycling, has been long recognized as necessary for shape changes accompanying migration of cells, it remained unclear whether any signaling interactions occurring specifically on endosomes were of importance for this process. One of the master regulators of cell migration is a small GTPase Rac which upon activation by GTP binding interacts with a number of effector proteins, leading to actin remodeling and formation of ruffles and lamellipodia [37]. The plasma membrane has been considered a primary residence of active Rac, with endocytic trafficking merely ensuring its proper targeting to cholesterol-rich, raft-like domains of the membrane [38]. Recently however, high-resolution time-lapse imaging employing a probe for detecting Rac-GTP or photoactivatable GFP-Rac enabled precise tracking of Rac activity and localization beyond the plasma membrane, on intracellular structures [35]. Activation of Rac on endosomes was demonstrated by measuring the membrane recruitment of a fluorescent probe that consists of Cdc42 and Rac interactive binding motif (CRIB) and, as such, specifically interacts with Rac-GTP. The process of Rac activation, attributed to the action of the Rac-specific guanine nucleotide exchange factor (GEF) Tiam1 was further shown to take place on endosomes by virtue of its dependence on Rab5- and clathrin-dependent endocytosis. The fate of endosomal active Rac was tracked using Rac fusion with photoactivatable GFP and two-photon excitation, and was shown to involve Arf6-mediated recycling of activated Rac toward specific cell surface domains characterized by formation of ruffles. These trafficking events were proposed to be crucial for the localized regulation of actin dynamics directed by Rac which promotes cell migration (Figure 2b). Accordingly, imaging of GFP-Rab5 dynamics in mammalian tumor cells in three-dimensional matrices and in developing zebrafish embryos indicated that this GTPase stimulates mesenchymal, Rac-controlled migration in different biological systems. Thus, the use of specific probes to trace the spatial dynamics of Rac activation paved the path to the elucidation of a previously unappreciated role for signaling endosomes in coordinating cellular events leading to cell migration.

Persistent cAMP signaling emitted from endosomes

Internalized G-protein coupled receptors (GPCRs) have been implicated in endosomal signaling involving MAP kinases via their interactions with β -arrestin adaptors [8]. However due to lack of tools to reliably detect the generation of second messengers evoked by intracellular GPCRs, it has remained unclear whether the ability of certain GPCRs to elicit cAMP-dependent signals via heterotrimeric G proteins is retained after internalization. This challenge has been overcome in a recent study by Calebiro and colleagues who developed tools and experimental models to visualize cAMP signaling upon TSH receptor stimulation in native mouse tissues [28]. The authors created a transgenic mouse expressing the FRET sensor for cAMP Epac1-camps (described above) and established three-dimensional cultures of thyroid follicles isolated from such mice. Placing the follicles under the laminar-flow perfusion chamber allowed rapid and controlled dosing of the TSH ligand during microscopical observations and FRET measurements. Interestingly, it turned out that TSH applications that were longer than 30 s were accompanied by an increase in the intracellular cAMP levels that lasted several minutes after TSH removal. In parallel, the authors have employed Alexa594-labeled bovine TSH as a novel tool to monitor ligand endocytosis and could observe that increased cAMP production correlated with the internalization of the

ligand. Further colocalization studies have demonstrated that in primary thyroid cells the ligand was present in tubulovesicular endosomal compartments where G-protein α -subunits and adenylyl cyclase III could be detected, arguing that cAMP signaling may be emitted also intracellularly. Blocking of TSH endocytosis by the dynamin inhibitor dynasore abolished sustained cAMP production after TSH removal. Moreover, the reorganization of actin cytoskeleton and the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), which are among the earliest effects of TSH on thyroid cells, were severely impaired upon blocking of endocytosis. These data, additionally supported by mathematical modeling of cAMP gradient production, led to the conclusion that persistent production of cAMP takes place from endosomes after internalization of TSH and its receptor. The findings represent a departure from the prevailing view that signaling of GPCRs to second messengers occurs at the plasma membrane and ceases upon receptor endocytosis. A crucial element in making these novel observations has been the application of a sensitive FRET-based detection system measuring second messenger changes in the native tissue, complemented by intracellular tracking of a fluorescent signaling ligand.

SARA endosomes in asymmetric cell division

It is recognized that signaling elicited by the Notch receptor and its membrane-bound ligands of the Delta/Serrate/Lag2 family is regulated by endocytosis [39]. Notch signaling controls several developmental processes some of which require the unequal partitioning of pathway components into two daughter cells via asymmetric cell division. Certain trafficking mechanisms contributing to such partitioning have been identified, mainly through studies using *Drosophila* sensory organ precursor (SOP) cells. Accordingly, a SOP pI cell divides asymmetrically generating two daughter cells termed pIIa and pIIb that display differential Delta-Notch signaling with pIIb being the signal-sending and pIIa the signal-receiving cell [39]. However, what remained unclear has been the fate of internalized Delta and Notch already in the parental SOP prior to mitosis. This question has been addressed by Coumailleau and colleagues who employed antibody-based probes and imaging techniques to track internalized ligands and receptors in living SOP cells during asymmetric division [40]. To this end, the authors used monoclonal antibodies against Delta and Notch, fluorescently labeled by Alexa FluorTM-tagged Fab fragments of secondary antibodies. These probes were applied to the dissected thorax of *Drosophila* pupae and the internalization of the respective antigens was imaged in four dimensions using spinning disc microscopy. This real-time analysis, complemented by studies of marker colocalization in fixed tissues, led to the finding that internalized Delta and Notch reach a particular subpopulation of early endosomes termed SARA endosomes. These endosomes are defined by the presence of a phosphatidylinositol-3-phosphate-binding protein SARA (Smad anchor for receptor activation), involved in Smad/TGF β signaling [19,41]. Further imaging of Delta- and Notch-containing SARA endosomes led to a striking observation that these vesicles are targeted to the mitotic spindle during SOP cell division and distribute preferentially to the pIIa daughter cell. Thus, the asymmetric delivery of Delta-Notch complexes via SARA endosomes could serve as a mechanism that specifies the activation of Notch signaling in pIIa and its suppression in pIIb (Figure 2c). This conclusion was corroborated by a series of follow-up experiments in which imaging analyses in living or fixed cells have demonstrated that the mistargeting of SARA endosomes during SOP division can cause abnormalities in cell fate specification of the daughters. Of note, signaling via SARA endosomes has been recently reported to regulate the interactions between the human osteoblasts and haematopoietic progenitor cells, arguing that in different systems these endosomes perform various signaling functions related to intercellular communication [42].

Conclusions

It is abundantly evident that our understanding of the function of endosomes as signaling hubs has dramatically improved as a consequence of the emergence of high-resolution imaging technologies. For most part, these technologies have been instrumental in documenting not only the residence of signaling complexes in endosomes but also the existence in endosomes of the machinery that controls the activation state of these complexes. Now that we are in a position to track endosomally-emitted signals, the stage is set to ask how these signals are integrated with their counterparts at different cellular sites. Addressing these questions will depend on future development of refined technical tools and conceptual models for analyzing complex spatio-temporal signaling patterns. In particular, the application of novel light microscopy imaging techniques which bypass the principal limitation of light diffraction and achieve super-resolution in the range of tens of nm [43], will likely lead to unprecedented insights into the signaling functions of endosomes.

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* of special interest

** of outstanding interest

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Figure 1. Methods used to detect the presence of internalized ligands, receptors and associated signaling molecules on endosomes. See text for more details and specific application examples.

Figure 2.

Examples of tools and observations discussed in the text which document signaling roles of endosomes. **(a)** The schematic representation of the Raichu probe, used to detect the active state of the Ras GTPase (upper panel). Only GTP-bound Ras is able to interact with the Ras-binding domain of Raf (Raf-RBD), causing a conformational change which brings CFP and YFP into close proximity resulting in FRET (lower panel). **(b)** The role of endosomes in polarized cell migration. Rab5-dependent endocytosis is required for the activation of Rac taking place on endosomes via the specific exchange factor Tiam1. Activated Rac is subsequently delivered via Arf6-mediated recycling to the plasma membrane, as a prerequisite for the local formation of actin-based migratory protrusions. **(c)** Asymmetric delivery of SARA endosomes during division of the pI precursor cell of *Drosophila* sensory organ. SARA endosomes containing internalized Delta-Notch complexes associate with the mitotic spindle and are preferentially targeted to one of the daughter cells (pIIa, a signal receiving cell).