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Effects of endocytosis on receptor-mediated signaling

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Abstract

Cellular mechanisms of membrane traffic and signal transduction are deeply interconnected. The present review discusses how membrane trafficking in the endocytic pathway impacts receptor-mediated signaling. Examples of recent progress are highlighted, focusing on the endocytosis-signaling nexus in mammals.

Introduction

Close relationships between endocytosis and receptor-mediated cellular signaling have been recognized since early investigations of ligand-induced down-regulation of epidermal growth factor receptors (EGFRs, reviewed in [1]), and the identification of endosomes as discrete membrane compartments containing internalized growth factors and activated growth factor receptors [2–5]. Subsequent studies have verified and extended this relationship in many systems, as reviewed previously (e.g., [6–9]). The present discussion seeks to minimize duplication by focusing on recent developments and restricting scope to results from mammalian systems.

We will begin with a brief review of mechanisms determining the molecular sorting of signaling receptors in endosomes, and the role of these mechanisms in modulating long-term cellular signaling responsiveness. We will then discuss the hypothesis that endosomes serve, additionally, as sites of active signal initiation. There are other interesting examples of intracellular signaling that do not require receptor endocytosis per se (such as nutrient sensing by endosomes); these are not discussed here but excellent reviews have appeared elsewhere (e.g., [10]).

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Endosomes as sorting stations determining long-term cellular signaling responsiveness

Endocytosis of signaling receptors is widely recognized to confer long-term homeostatic control on cellular signaling responsiveness by adjusting the total cellular receptor complement, or surface-accessible complement, in accord with the cell's history of cognate ligand exposure or overall activation state. Ligand-induced activation typically increases receptor endocytic rate, and internalized receptors engage molecular sorting machineries that specify subsequent transport via divergent lysosomal and recycling routes. These events, in turn, determine the degree to which cellular ligand responsiveness is attenuated ('down-regulated') or sustained ('re-sensitized') under conditions of prolonged or repeated ligand exposure.

Many signaling receptors internalize via clathrin-coated pits and a considerable amount is now known about this mechanism (reviewed in [11]). However, it has been recognized for many years that additional endocytic mechanisms exist [12], and one area of recent progress is toward identifying alternate mechanisms relevant to signaling receptors. One that has been described recently requires endophilin but not clathrin, and is called 'fast endophilin-mediated endocytosis' (FEME) to distinguish it from clathrin-mediated endocytosis (CME) [13]. FEME is outwardly similar to CME in that dynamin and local actin polymerization contribute to endocytic membrane scission, but FEME occurs through the formation of distinct tubulovesicular structures lacking clathrin, with endophilin providing the major force for membrane deformation [14]. FEME also differs from CME in its mechanism of cargo selection. CME is generally engaged by receptor association with clathrin adaptor proteins [11], whereas FEME appears to be engaged by binding of proline-rich sequences in the receptor to the SH3 domain of endophilin [13]. Identification of the FEME mechanism is an exciting development and a remarkable number of signaling receptors appear to engage it, but questions remain. For example, the D4 dopaminergic receptor (DRD4) is a putative FEME cargo but its SH3 domain-interacting sequences were found previously to inhibit, rather than promote, endocytosis of receptors. In addition, mutating these motifs to fully destabilize SH3 domain binding results in a ligand-independent endocytic phenotype [15]. These observations, not easily reconciled with the present understanding of cargo engagement with FEME, suggest that still more remains to be learned about diversity and specificity in mechanisms of signaling receptor endocytosis.

Progress has also been made recently toward more fully understanding how signaling receptors are sorted after endocytosis. Ubiquitin-directed engagement of the endosomal sorting complex required for transport (ESCRT) is an important mechanism driving lysosomal down-regulation and is highly conserved, including in yeast where many components of this machinery were first identified [16]. However, it has been suspected for some time that additional mechanisms operate in higher eukaryotes. This appears particularly likely for the GPCR family, which is ~1000-fold more diverse in mammals than in yeast.

Early evidence suggesting the existence of additional endosomal sorting machinery emerged through the study of GPCR down-regulation leading to identification of a putative 'GPCR-

associated sorting protein' (GASP) that binds various GPCR cytoplasmic tails without requiring ubiquitination [17]. GASP-1 (or GPRASP1) is the founding member of a small protein family that is widely expressed in mammals but not found in yeast [18]. The precise cellular function(s) of GASPs remain poorly understood, but recent studies suggest interesting possibilities. GASP-1 binds Beclin2, a mammalian-restricted paralogue of Beclin1 (ATG6), through a Beclin2-specific N-terminal domain [19]. Beclin2 is otherwise similar to Beclin1, including in its ability to regulate the endosomal type III PI3-kinase (VPS34) and bind ATG14 that functions as a tethering protein in autophagosome-endolysosome fusion [20]. GASP-1 can also bind dysbindin as well as the stimulatory heterotrimeric G protein, Gs. These interactions appear to promote GPCR degradation by engaging the ESCRT through additional association with HRS, providing a path of alternate receptor connectivity to the ESCRT that does not require ubiquitination and is regulated by heterotrimeric G protein [21,22].

Studies of GPCR recycling provided further evidence for additional mechanisms of signaling receptor sorting in mammals. A PDZ and PX domain-containing protein called sorting nexin 27 (SNX27) was identified as a key protein that binds beta-adrenergic receptors in endosomes and promotes receptor recycling [23]. SNX27 associates with the WASH - Arp2/3 actin nucleation complex and this interacts, in turn, with the retromer complex. This 'actin module-SNX27-retromer tubule module' (ASRT) machinery assembles at the base of specialized membrane tubules that extend from the endosome limiting membrane and mediate cargo exit from endosomes [24]. SNX27 interacts not only with WASH but also with retromer directly through the arrestin-like protein VPS26, and the integrated ASRT machinery appears to mediate specific endosome-to-plasma membrane transport of various signaling receptors as well as other specialized membrane cargoes such as the Glut1 (SLC2A1) glucose transporter [25]. Physiological roles of this mechanism are only beginning to be explored but are likely considerable. For example, the ASRT machinery was shown recently to mediate a discrete route of localized membrane delivery to the postsynaptic plasma membrane that is required for functional surface expression of excitatory neurotransmitter receptors at synapses [26]. It is also interesting to note that human genetic studies have linked core components of retromer, as well as SNX27 and WASH components, to neurological and neurodegenerative syndromes (reviewed in [27]).

Endosomes as sites of receptor-mediated signal initiation

As noted above, it was proposed from the earliest investigations that endosomes may themselves function as active signaling sites. This idea, formalized in the 'signaling endosome' hypothesis, has been supported by many subsequent studies. However, two fundamental questions remain incompletely resolved. First, are endosomes bona fide sites of significant signal initiation under normal physiological conditions? Second, does the endosome signal confer functional effects different from the plasma membrane signal?

Perhaps the strongest support, overall, for an affirmative answer to both questions comes from the study of retrograde neurotrophin signaling (reviewed in [7]). Sympathetic neurons require stimulation by ligands released from peripheral targets that they innervate. Absent such signals, neurons undergo apoptotic cell death and are eliminated. One trophic signaling

ligand, nerve growth factor (NGF), is a polypeptide that activates the TrkA tyrosine kinase receptor. TrkA activity is required to induce an anti-apoptotic transcriptional program mediated by a downstream MAP kinase cascade. Using a compartmentalized culture system, NGF applied selectively to distal axons was shown sufficient to elicit an anti-apoptotic transcriptional response in the neuronal cell body. Further, NGF was shown to internalize together with TrkA, and endosomes containing both cargoes were shown to move from the axon to cell body. The current model is that TrkA is continuously ligand-activated and phosphorylated in signaling endosomes during retrograde movement, effectively carrying the trophic signal from the cell periphery to nucleus. Considerable evidence supports this model, including recent work elaborating features of cytoskeletal control that are required for both retrograde endosome movement and the functional trophic signaling response [28]. However, this model is not beyond reproach. For example, some results suggest that chemical inhibition of TrkA kinase activity in the cell body is not sufficient to block the trophic signal initiated in distal axons; thus it has been suggested that the retrograde signal may be carried by a downstream kinase rather than by internalized TrkA itself [29].

Evidence regarding endosome signaling by other receptors is similar in broad outline, if less compelling in some of its detail. A number of studies followed the development of a mutant dynamin construct that inhibits clathrin-mediated endocytosis when over-expressed. It was first shown that this construct blocked activation of the ERK MAP kinase elicited by EGFRs [30]. Then similar results were reported for ERK activation elicited by several GPCRs, starting with beta-adrenergic receptors [31]. Such results were interpreted initially as direct evidence of receptor signaling from endosomes. Refuting this claim, mutant dynamin was later found to block ERK activation elicited by an endocytosis-defective GPCR [32] as well as receptor-independent activation of protein kinase C [33]. These observations suggested that dynamin has additional signaling-relevant effect(s), such as internalizing an essential downstream mediator or pathway regulator separate from receptors [32,33], but also highlighted the inherent limitations of indirect methods when applied to determining the subcellular location of key signaling reactions. This limitation persists in more recent studies as well. Still the accumulated weight of evidence, particularly with the development of more direct approaches (discussed later in this review), favors the hypothesis that some signaling reactions indeed occur in endosomes. Attention has shifted to the second question, whether endosome-initiated signals are functionally different from those emanating from the plasma membrane.

Wingless or Wnt signaling provides a particularly interesting example because different mechanisms appear to operate at each location [34]. A key event in canonical Wnt signaling is the ligand-dependent reduction of cytoplasmic GSK3 activity, which reduces the rate of beta-catenin turnover and subsequently drives the downstream transcriptional response in receiving cells. Endosomes were proposed to act as signal-activating devices by physically sequestering GSK3 from the cytoplasm into intralumenal vesicles [35]. This idea was been questioned since its proposal because previous studies showed that a portion of the Wnt receptor complex (low density lipoprotein receptor-related protein 6) can act directly as a pseudosubstrate inhibitor of GSK3 kinase activity [36]. Recent work may have resolved this conundrum by suggesting that both mechanisms are germane, with pseudosubstrate

inhibition rapidly reducing cytoplasmic GSK3 activity from the plasma membrane and GSK3 sequestration in endosomes mediating a sustained component of the response [37].

A number of other recent studies provide interesting suggestions regarding the functional significance of endosome signaling. One study described biphasic EGFR signaling determined by the concentration of growth factor to which cells are exposed. At low ligand concentration, downstream MAP kinase signaling is weak and mediated by activated EGFRs present in the plasma membrane. At higher ligand concentration, receptors are ubiquitinated and internalized. This results in a supra-linear increase in downstream signaling strength by redistributing activated receptors to endosomes, where downstream signaling is more efficient [38]. Another study reported that chemically distinct polypeptide ligands can elicit different responses via the EGFR through ligand-specific differences in endosomal dynamics, and that endosomes can be considered ‘quantal’ signaling devices because they contain a relatively uniform number of activated receptors [39]. A third interesting example is a study of MAP kinase signaling initiated by GPCRs. Here, it was proposed that distinct spatiotemporal profiles of MAP kinase activation can be produced by receptor recycling through different endosome populations [40].

Recent progress in the study of endosome signaling and its consequences

Significant advances have been made recently toward determining the subcellular location of defined receptor-mediated signaling mechanisms directly. As noted above, this is a key limitation of many studies in this area, and directly detecting signal initiation would seem feasible for growth factor signaling because pathway activation is associated with receptor phosphorylation and physical association of receptors with a signaling adaptor. Indeed, energy transfer methods have yielded arguably direct evidence of receptor-adaptor association in endosomes (e.g., [41]). However, concerns have been raised regarding the degree to which such interactions occur under conditions of physiological (versus supra-physiological) protein expression and ligand concentration [6]. Recently gene editing has been used to express recombinant proteins at near-endogenous levels. The results support endosome-based signaling under physiological or near-physiological conditions, and over a wide range of ligand concentration. They also suggest that endosomes make a remarkably large contribution to overall EGFR signaling activity, apparently more than that emanating from the plasma membrane [42].

Determining the subcellular location of canonical GPCR signal initiation poses an additional challenge because this is a catalytic reaction, involving formation of an activated receptor-G protein complex that is very short-lived under physiological conditions [43]. Recent progress on this problem emerged through development of single domain antibody fragments (nanobodies) that recognize specific GPCRs or G proteins in a conformation-selective manner. These reagents, generated initially for in vitro structural studies [44], were adapted to act as ‘conformational biosensors’ of GPCR and G protein activation when expressed as cytoplasmic fusion proteins.

Using such nanobody-derived tools, an activated conformational state of the beta-2 adrenergic receptor and a conformational intermediate in the process of cognate G protein

(Gs) activation were detected within seconds after application of agonist ligand at the plasma membrane, consistent with the conventional model. In addition, evidence of a discrete activation phase was detected at endosomes, commencing within minutes after the arrival of internalized receptors and persisting after the initial plasma membrane activation phase diminished [45].

By adapting a light-activated adenylyl cyclase enzyme to optogenetically drive production of the second messenger cyclic AMP (cAMP) selectively from the plasma membrane or from the endosome limiting membrane, it was then shown that endosome-based signal activation preferentially induces cAMP-dependent transcriptional responses relative to signal initiation from the plasma membrane [46]. Caveats remain (e.g., protein over-expression and potential perturbing effects of biosensors or optogenetic actuators) but, together, these results constitute arguably direct evidence of GPCR-mediated signal initiation from endosomes and provide initial insight to its functional significance.

Outlook

Accumulating evidence strongly supports the general idea that receptor-mediated signaling and membrane trafficking processes are intimately interconnected (Figure 1). One connection, which is now well established, is that endocytic trafficking modulates long-term cellular responsiveness by dynamically adjusting the number of receptors accessible to extracellular ligands in the plasma membrane (or relevant domains thereof, such as synapses). Multiple endosomal sorting machineries contribute to such control, at least in higher eukaryotes, and these remain to be fully elaborated. Because some signaling receptors can engage more than one sorting machinery and ‘switch’ itinerary [47], much also remains unknown about how discrete endosomal machineries are coordinated and regulated to execute the appropriate net sorting decision.

A second connection in the signaling-endocytosis nexus, which has been proposed for many years but largely through indirect or correlative evidence, is that endosomes serve as sites of active signal initiation. There is now direct evidence that growth factor receptors engage key signaling adaptors in endosomes under physiologically relevant conditions, and that endosomes are major contributors to the net cellular response. It is likely that GPCRs signal analogously, using arrestins as alternate signaling scaffolds [48]. Moreover, as summarized above, there is now arguably direct evidence that canonical GPCR - G protein activation occurs in endosomes.

A natural next question is how endosome-based signaling is controlled and terminated. Proteolytic destruction of receptors in the endolysosome system is one obvious mechanism based on first principles, and there is considerable evidence that this underlies long-term down-regulation of many cellular signaling responses [47]. Another well-established mechanism is through ubiquitin-directed transfer of receptors from the limiting endosome membrane to intralumenal vesicles. This is opposite to endosome sequestration of GSK3 that is proposed to activate Wnt signaling, as discussed above. However, physical sequestration of receptors into intralumenal vesicles has long been recognized to terminate growth factor signals prior to receptor proteolysis [16]. A third possible mechanism is

through endosome acidification, a process that has long been recognized to promote ligand-receptor dissociation and modulate growth factor responses [4,49]. It is presently thought that endosome acidification terminates some receptor-mediated signals (e.g., [50]) but not others (e.g., [42]). Arrestins likely also function in endosome signal termination, particularly of signals initiated by GPCRs to which arrestins directly bind. However, arrestins and arrestin-like proteins likely also have distinct trafficking and signaling functions that include promoting some cellular signals (as noted above and reviewed authoritatively in [48]).

Finally, we note that much of our current understanding is derived from study of simplified experimental models. Thus an important direction for future research is to delineate relationships between signaling and endocytosis as they exist in native systems. The physiological and therapeutic implications of such relationships are only beginning to be explored and likely significant. For example, the phenomenon of functional selectivity or agonist bias, now a major focus in GPCR-targeted therapeutics (see [51]), was recognized in large part through ligand-specific effects on GPCR endocytosis (reviewed in [52]). We anticipate that further elucidation of the role of endosomes as sites of both molecular sorting and signaling, and elucidating how chemically diverse ligands affect these functions, will provide fundamental biological insight useful for developing improved therapeutics and directing the actions of existing drugs more precisely.

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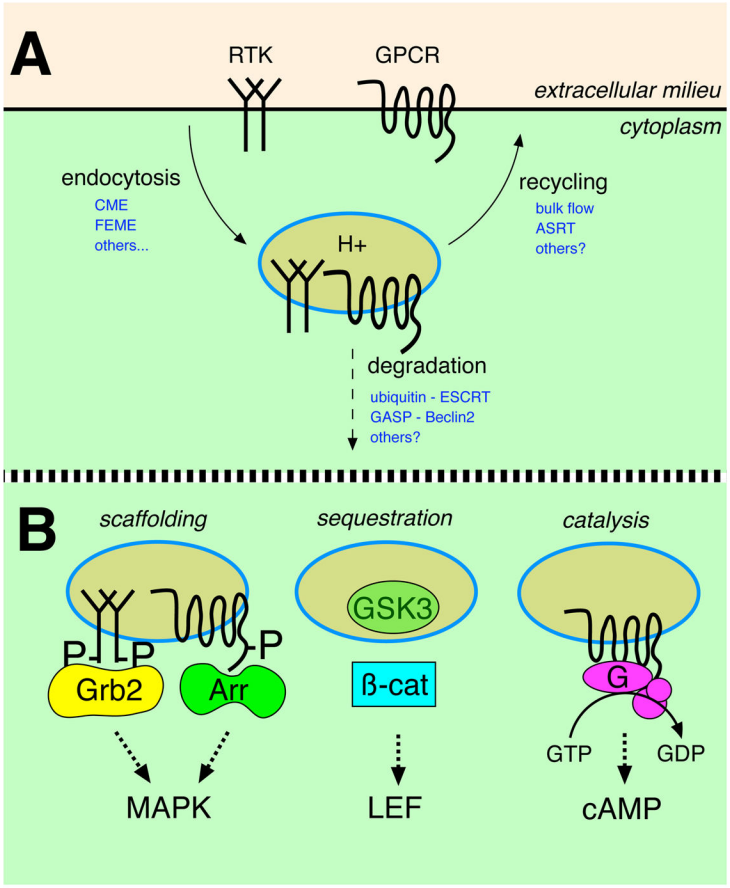

Figure 1. Simplified schematic of endocytic trafficking itineraries relevant to signaling receptors and proposed mechanisms of endosome-based signal activation

(A) Signaling receptors such as receptor tyrosine kinase (RTK) growth factor receptors and members of the large G protein-coupled receptor (GPCR) family can internalize by various routes, including clathrin-mediated endocytosis (CME), fast endophilin-mediated endocytosis (FEME) and probably others. Internalized receptors are sorted by various mechanisms, a subset of which are indicated in the diagram using abbreviations explained in the text. These mechanisms determine the degree to which internalized receptors are delivered to lysosomes (degradation) or returned intact to the plasma membrane (recycling), divergent itineraries that down-regulate or sustain / resensitize net cellular signaling responsiveness, respectively. **(B)** Three biochemical principles that are currently thought to underlie signaling from endosomes. ***Scaffolding***: Growth factor receptors can engage signaling adaptors such as Grb2 in the endosome limiting membrane, driving downstream activation of MAP kinase (MAPK) modules. GPCRs likely signal from endosomes in an analogous manner, except using arrestins or beta-arrestins (Arr) as alternate signaling scaffolds. ***Sequestration***: Wnt / Wingless signaling is proposed to occur from endosomes through physical sequestration of GSK3 into the endosome lumen. This reduces cytoplasmic GSK3 activity, stabilizing and promoting cytoplasmic accumulation of beta-catenin (β-cat) that functions as a downstream mediator of transcriptional signaling through binding to the transcription factor LEF. ***Catalysis***: GPCRs can activate heterotrimeric G proteins (G) from

the endosome limiting membrane, which promotes downstream signaling through production of cytoplasmic second messenger molecules such as cyclic AMP (cAMP).