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Endocytosis and Signaling: An Inseparable Partnership

Minireview

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Endocytosis through clathrin-coated pits has long been regarded as a mechanism to switch off plasma membrane receptor signaling. In this view, trapping of receptors into coated pits is considered mainly a passive phenomenon that occurs via endocytic motifs exposed by receptors, either constitutively or following interaction with ligands. Growing evidence, however, suggests an important cross-talk between endocytosis and signaling. In this review, we will focus on some of the recent advances on the molecular mechanisms that connect the machinery of receptor-mediated signaling and endocytosis via clathrin-coated pits.

Signaling from Endosomes and Its Relevance

While it is clear that active receptors signal from the plasma membrane, they are frequently still in their active state in endosomes, suggesting continuation of signaling from this intracellular compartment. Initial studies with immobilized ligands and internalization-impaired receptors did not generally support a critical role for signaling in the endocytic pathway. Recently, however, renewed interest on this topic was stimulated by the availability of molecular tools to inhibit clathrin-mediated endocytosis in general, or, selectively, the internalization of specific receptors. These include dominant-negative mutants of the GTPase dynamin (a protein required for the fission of clathrin-coated vesicles from the plasma membrane) or of proteins that bind clathrin and the endocytic clathrin adaptor AP-2, or endocytic motifs of receptor subclasses (Miller and Lefkowitz, 2001; Slepnev and De Camilli, 2000). Several studies have thus revealed a role for endocytosis in the propagation of intracellular signals. In the presence of dominant-negative mutants of dynamin or β-arrestin, reduced activation of the extracellular signal-regulated kinases (ERKs) was detected, following activation of receptor tyrosine kinases (RTKs) and many G protein-coupled receptors (GPCRs). Decreased insulin-dependent PI3-K activity was also reported. The field has been covered recently by some excellent reviews (see, for instance McPherson et al., 2001; Miller and Lefkowitz, 2001); thus, we will restrict our discussion to the possible physiological relevance of signaling from the endosome.

Given the importance of ERKs (and PI3-K) in signaling, one would predict dramatic effects of the blockade of endocytosis on biological phenotypes. However, in the few cases where biological end points were measured, inhibition of endocytosis did not result in attenuation of biological effects (Ceresa et al., 1998; Vieira et al., 1996), arguing against a general relevance of endosome-originated signals. This contribution becomes essential when signals generated at the cell periphery must be translocated over long distances, as in retrograde axonal signaling. In this case, intracellular signaling cannot be achieved by diffusion and the transport of membrane-bound signaling complexes represents an efficient way to deliver the signal(s) to a physiologically relevant location within meaningful time scales. For example, neurotrophic factors and their cognate Trk receptors are retrogradely transported from nerve terminals to cell bodies, where they activate the transcriptional machinery. The compartmentalized application of noninternalizable NGF reveals how endocytosis is not required for signaling from the plasma membrane of the cell bodies to the nucleus, but is absolutely necessary for the effects of NGF applied to nerve terminals. Application of NGF to distal axons is sufficient to promote survival, an event that requires NGF-induced PI3-K activity (Kuruvilla et al., 2000). Two different pools of active PI3-K seem involved in the process. The nerve terminal pool may be involved in initiating retrograde transport. A second pool, likely generated from active endosomal TrkA, is required in the cell bodies (Kuruvilla et al., 2000). Thus, the same signaling pathway downstream to a receptor may exert different functions depending on the topographical origin of the signal.

The Endocytic Pathway as the Switch-off for Receptor Signaling

Irrespective of whether signaling continues after endocytosis, the end point of the endocytic journey of activated receptors is to undergo inactivation. This may occur by several mechanisms, from dissociation of the ligand at the acidic pH of endosomes and subsequent cell surface recycling to receptor degradation in lysosomes. For receptors whose fate is the lysosome, e.g., RTKs, there is evidence that regulation of this step may affect the duration of the stimulus. In this connection, a growing area of investigation concerns the role of ubiquitination. Protein ubiquitination, once thought to be involved selectively in the proteosomal destruction of soluble proteins, has now also been implicated at multiple steps of the endocytic pathway, from the internalization reaction to the maturation of endosomes and lysosomal delivery. Both receptors and cytosolic endocytic proteins can be ubiquitinated. Impairment of their ubiquitination delays lysosomal degradation and may lead to prolonged receptor signaling. For example, mutations that impair the property of c-Cbl (an E3 ubiquitin-conjugating enzyme) to induce the ubiquitination of growth factor receptors have oncogenic properties

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(Levkowitz et al., 1998). It is also of interest that impairment of the function of TSG101/Vps23, a protein that contains a catalytically inactive E2 ubiquitin-conjugase domain, perturbs endosomal trafficking (Babst et al., 2000) and induces cell transformation. Some of the effects of defective TSG101/Vps23 function on cell proliferation may be mediated by the property of this protein to act as a dominant-negative modulator of a ubiquitination pathway controlling MDM2/p53 levels (Li et al., 2001). However, additional effects mediated by perturbation of late endosomal traffic, possibly by inhibition of the formation of multivesicular bodies (Babst et al., 2000), cannot be excluded.

One interesting example of regulated receptor internalization and degradation that is independent of the ligand itself is reported in a study on Wingless signaling in *Drosophila* embryos published in the June 1 issue of *Cell* (Dubois et al., 2001). Wingless (the fly homolog of mammalian Wnt1) is an extracellular ligand that is secreted along transversal stripes in the embryo and initially diffuses equally at the posterior and anterior side of the stripes. As the embryo develops, however, the localization of receptor-bound Wingless at the two sides of the stripes becomes asymmetric due to enhanced lysosomal degradation of the ligand-receptor complex at the posterior side. Decreased Wingless signaling, in turn, has important effects on cell fate in the neighboring cells. Downregulation of lysosomal delivery, achieved genetically by reduction of the function of clathrin or of the deep-orange protein, prevents the posterior decrease in Wingless signaling and the corresponding developmental changes. Similar effects are produced by an impairment of endosomal maturation induced by chloroquine, which blocks endosome/lysosome acidification. Therefore, regulation of the endosomal routing of ligand-activated receptors is differentially controlled by the tissue microenvironment in different cells. This defines a new paradigm for the spatial and temporal regulation of signaling in tissue differentiation. Of additional interest is the finding that the enhanced degradation of Wingless and of its receptor is dependent on the activation of the EGF receptor (EGFR) signaling pathway, underscoring the relevance of signaling events to the regulation of endocytosis, an issue that will be further discussed below.

Different Programs for Different Pits?

A complex scenario of how endocytosis is regulated by signaling is emerging. An interesting example is provided by the properties and mechanism of action of Rab5, which, in addition to its function in early endosomal trafficking, plays a role in endocytosis at the coated pit level. As all small GTPases, Rab5 cycles between an active GTP-bound and an inactive GDP-bound state through the opposing actions of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Rab5 function is controlled by signals emanating from RTKs, via activated Ras. Active RTKs can activate the Ras-specific GEF Sos1. This causes an increase of the intracellular levels of Ras-GTP, which in turn binds to several intracellular transducers, thus leading to the propagation of signals. Among the Ras-GTP effectors is RIN1, a protein containing a signature domain of Rab5-family GEFs, the Vps9 domain. In a paper published in the July issue of *Developmental Cell*, Tall et al. demonstrate that RIN1 does indeed have Rab5-specific GEF activity (Tall et al., 2001). Binding of Ras-GTP to RIN1 potentiates its GEF activity on Rab5. Accordingly, overexpression of RIN1 stimulates internalization of the EGFR and endosome fusion. The deactivation loop seems equally controlled by signaling. The RN-tre protein is a Rab5-specific GAP, whose overexpression results in the attenuation of receptor endocytosis, consistent with the notion that it reduces the levels of active Rab5 (Lanzetti et al., 2000). Interestingly, the activity of RN-tre is also regulated in vivo, in a negative fashion, by active EGFRs. Thus, signals emanating from active RTKs control both activation and deactivation of Rab5 and hence regulate receptor internalization (Figure 1).

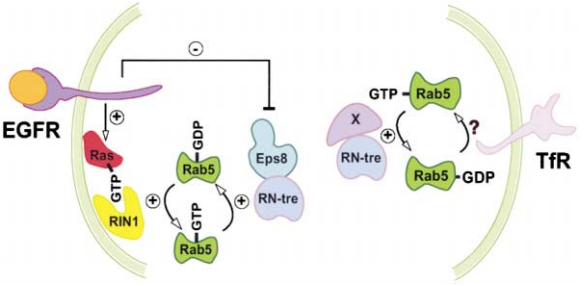

Figure 1. Rab5 in Ligand-Induced and Constitutive Endocytosis

Active GTP-bound Rab5 stimulates internalization of both the EGFR and the TfR. Left: For EGFR, the molecular circuitry involved is depicted. Right: In the case of the TfR, the GEF activity involved remains unknown. A hypothetical X protein might substitute for eps8 in allowing the GAP activity of RN-tre. Details are in the text.

These results, as well as other recent studies, shed light on another important issue, the cargo heterogeneity of endocytic clathrin-coated pits and the existence of specific molecular machineries that regulate the internalization of different receptors. Perturbation of the function of RIN1 affected the internalization of the EGFR (a ligand-induced reaction), but not constitutive endocytosis of the transferrin receptor (TfR) (Tall et al., 2001). Similarly, the action of RN-tre was dependent on its interactor eps8 in the endocytosis of the EGFR, but not in the endocytosis of the TfR (Lanzetti et al., 2000 and Figure 1). In another setting, it was found that tyrosine phosphorylation of Eps15, an accessory endocytic protein, was required for EGFR but not for TfR internalization (reviewed in McPherson et al., 2001). However, Rab5 and Eps15 are required for endocytosis of both the EGFR and the TfR. A multilayered circuitry of regulation might exist, in which common components of the endocytic machinery, such as Rab5 and Eps15, are controlled in a "pit and/or receptor-specific" fashion. Thus, although several studies have indicated that constitutive recycling receptors and ligand-regulated receptors can be present in the same endocytic clathrin-coated vesicle, these organelles can be quite heterogeneous in membrane cargo and, presumably, in adaptor proteins. Heterogeneity may apply both to the mechanisms that nucleate clathrin coats as well as to those that target specific proteins to the coated pits (specific adaptors recruit specific sets of proteins). Whatever the case, this differential regulation points to potentially interesting targets for therapeutic interventions aimed at selectively

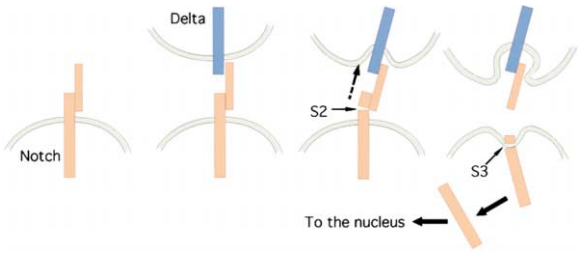

Figure 2. Trans-Endocytosis and Activation of Notch

Notch is depicted on the plasma membrane as a heterodimer resulting from the S1 cut. After interaction with Delta, endocytosis starts in the Delta-expressing cell, imposing a molecular strain on Notch (dashed arrow) that allows for the occurrence of the S2 cut. The S3 cut then ensues in a process that might require endocytosis in the Notch-expressing cell. Details are in the text.

affecting a subset of clathrin-mediated internalization reactions.

Endocytosis Activates Signaling

Beyond a role in signal termination and signal propagation, endocytosis might have a function in receptor activation, as in the case of Notch, a receptor required for correct specification of cell fate. The biogenesis of Notch (Figure 2) relies on three sequential cleavages (S1, S2, and S3). S1 occurs in the extracellular portion of Notch, yielding a heterodimeric complex, and takes place in the Golgi. In response to interaction with its ligands (including Delta, a transmembrane protein expressed by neighboring cells), Notch undergoes S2 in the extracellullar juxtamembrane region. S2 is a prerequisite for S3, a cleavage in the transmembrane region that releases the intracellular domain, thus allowing its translocation to the nucleus, where it acts as a transcriptional regulator (see Parks et al., 2000 and references therein).

In *Drosophila*, reduction in the function of shibire, the fly homolog of dynamin, results in phenotypes remarkably similar to those of Notch loss-of-function. Accordingly, shibire (and thus presumably endocytosis) is required for Notch activation. Unexpectedly, endocytosis in both the Delta- and Notch-expressing cells seems to be necessary (see Parks et al., 2000, and references therein). Endocytosis of Delta is accompanied by endocytosis of the extracellular domain of Notch in the Delta-expressing cell, a process known as Notch trans-endocytosis (Parks et al., 2000). Impairment of endocytosis in the Delta-expressing cell results in the attenuation of Notch trans-endocytosis and Delta-dependent signaling in the Notch-expressing cell. Thus, the extracellular domain of Notch may have an inhibitory function on Notch signaling, and Notch trans-endocytosis may serve to remove such inhibition. How this disinhibition leads mechanistically to further processing is unknown. One proposed scenario suggests that the molecular strain imparted on the Notch/Delta complex during the initial phases of endocytosis by the Delta-expressing cell causes a conformational change in Notch that allows the S2 cleavage to occur (Figure 2).

Why endocytosis is also required in the Notch-expressing cell is not known. However, a scenario can be suggested by analogy to a model derived from studies of the β-amyloid precursor protein (APP). APP is also processed through sequential cleavages, the final cleavage (like the S3 of Notch) being performed by γ-secretase. It is now clear that, both for Notch and APP, presenilin(s) is required at this step (see, for instance, Struhl and Adachi, 2000). Complete processing of APP appears to require internalization, a possibility also supported by the recent report of an endosomal pool of presenilin. Although the precise subcellular distribution of presenilin remains somewhat controversial, one can postulate a similar scenario for Notch, with S2 cleavage followed by internalization and routing of the receptor to endosomes, where the S3 cleavage occurs. Since late endosomes are transported toward the central region of the cell via retrograde microtubular motors, delaying cleavage to a post-internalization step may facilitate delivery of the final proteolytic fragment of the Notch receptor to the nucleus.

Convergence of Molecular Machinery in Signaling and Endocytosis

Given the functional links between endocytosis and signaling, it is not surprising that many cytosolic proteins function in both processes. For example, Grb2 and other signaling adaptor proteins, such as Shc or Crk, also bind, directly or indirectly, dynamin and/or other accessory factors of clathrin-mediated endocytosis. A well studied example of a protein with a dual function in signaling and endocytosis is represented by β-arrestin, which binds activated GPCRs and terminates their interaction with heterotrimeric G proteins. In addition, β-arrestin binds clathrin, the clathrin adaptor AP-2, and membrane phosphoinositides, thus participating in the assembly of coated pits and in the coupling of GPCR activation to their endocytosis. Finally, β-arrestin binds Src and components of the MAPK pathway and functions as an activator of this pathway (Miller and Lefkowitz, 2001). Many other proteins act as multifunctional adaptors among membrane proteins to be internalized, clathrin coat proteins, the membrane bilayer, and signaling networks. These include Eps15 and Eps15R, intersectins, amphiphysin, Numb-like proteins, epsins, AP180/CALM and other ENTH of VHS domain-containing proteins (reviewed in McPherson et al., 2001; Slepnev and De Camilli, 2000).

Another interesting example is provided by dynamin, which has been recently implicated in signaling functions that are independent of endocytosis (Fish et al., 2000; Whistler and von Zastrow, 1999). In particular, overexpression of dynamin 2 induces apoptosis by a mechanism that does not involve alterations in endocytic trafficking and requires p53, possibly by activating its transcriptional activity (Fish et al., 2000). In addition, an important, albeit unclear, connection between dynamin and the actin cytoskeleton has emerged, suggesting a potential signaling function of this GTPase on actin nucleation and dynamics, including on a pool of actin which may participate in the endocytic reaction. Some of the SH3 domain-containing partners of dynamin interact with the polyphosphoinositide phosphatase synaptojanin, components of the Ras and Jnk signaling pathways, and with proteins that control nucleation of the actin cytoskeleton such as WASP and Rho family members. Most of these proteins dimerize or have multiple SH3 domains, thus facilitating generation of

signaling complexes. Most importantly, formation of these complexes requires activation of the receptor (McPherson et al., 2001; Slepnev and De Camilli, 2000).

While it is evident that we have only begun to scratch the surface of these intricate networks, the overlap between the molecular machineries of endocytosis and signaling clearly represents an efficient mechanism through which receptors can control both downstream signaling and their own fate.

Abnormal Balance between Endocytosis and Signaling in Diseases

All of the above evidence predicts a role for endocytic proteins in pathological conditions where the balance between stimulation and attenuation of signaling is subverted, the first and foremost of which is cancer. Recent discoveries in this area include the link between trasformation and growth factor receptor degradation by ubiquitination (see above), as well as the identification of the unexpected involvement in endocytosis of the putative tumor metastasis suppressor protein nm23H1. nm23H1 is a nucleoside diphosphate kinase (NDK), i.e., an enzyme that synthesizes nucleoside triphosphates from the respective diphosphates. Recently, genetic evidence was reported that indicates that the NDK activity of Awd, the *Drosophila* homolog of nm23, is required for dynamin-dependent endocytosis (Krishnan et al., 2001). Awd might function in endocytosis by generating the GTP pool required for dynamin function and facilitating GTP loading (and hence activation) of this GTPase, which has an unusually low affinity for GTP. Impairment of nm23 function might therefore result in prolonged receptor exposure at the plasma membrane and sustained signaling. A further twist to the story is provided by the report that nm23H1 interacts with and inactivates Tiam1, a GEF that turns on the small GTPase Rac in the signaling cascade initiated by surface receptors (Otsuki et al., 2001), suggesting that lack of nm23 function might result in enhanced Rac-dependent signaling. Thus, nm23H1 appears to intersect pathways controlling endocytosis and actin dynamics through two distinct GTPases. The "authentic" suppressor nature of nm23 is not established yet, but, as speculated here, its abnormal function could represent a paradigm for how interference with the intricate network of connections between endocytic and actin remodeling machineries may lead to neoplasia.

As interfaces between endocytosis and signaling are being elucidated, proteins with a dual role in these processes are being implicated in other diseases. An interesting example concerns receptors of the LDL receptor superfamily, which includes receptors involved in brain development and function. Some of the adaptor proteins that recognize the cytoplasmic endocytic motifs of members of this receptor family have now been identified. Dab1, one such adaptor, participates both in endocytosis and signaling, and its abnormal function affects neuronal migration in the mammalian brain (Herz, 2001; Morris and Cooper, 2001), forecasting possible future links between alterations of endocytic/signaling proteins and diseases of neurological interest.

Conclusions

Far from being a simple attenuator, endocytosis is now also implicated in the initiation and propagation of signals. Signaling reciprocates by controlling endocytosis and contributing to its ability to selectively sort receptors. It appears that the quiet days of endocytosis as a relatively "stand-alone" process are over, as we expose the intricate network that connects it to actin, membrane dynamics, lipids metabolism, signaling adaptors, and transducers. Phenotypically, this is mirrored by an important role of endocytosis in proliferation, differentiation, cell survival, neurotransmission, embryogenesis, and cell fate specification. An emerging role of alterations of the endocytic machinery in diseases is therefore predicted.

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