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Endocytosis and intracellular sorting of receptor tyrosine kinases

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1. ABSTRACT

Binding of the peptide growth factors (GF) on their surface receptors triggers rapid endocytosis of ligand-receptor complexes via clathrin-coated pits. Internalized receptors pass through the endosomal compartment where they are efficiently sorted to the lysosomal degradation pathway. Accelerated internalization and degradation of GF receptors leads to dramatic down-regulation of the receptors. Hence, we discuss the pathways, molecular mechanisms and the biological role of the GF-induced receptor endocytosis and lysosomal targeting. The endocytosis of epidermal growth factor (EGF) receptor is employed as a model system to describe the role of clathrin, clathrin adaptors and other proteins in the recruitment of receptors into coated pits and in the endosomal sorting process.

2. INTRODUCTION: RECEPTOR-MEDIATED ENDOCYTOSIS

Many macromolecules enter into the cell by the process of receptor-mediated endocytosis (1). The ligand-receptor complexes formed on the cell surface can be selectively recruited into clathrin-coated pits, small areas of the plasma membrane covered with the clathrin coat from the cytoplasmic side of the membrane (2). Coated regions of the membrane provide a mechanical basis for rapid vesicle budding as they can invaginate inward and capture the membrane to pinch off vesicles. Clathrin-coated pits and vesicles contain two major structural components: clathrin itself and adaptor protein complexes, APs. Clathrin consists of three copies each of heavy chain (~190 kD) and light chain (~23-27 kD), forming a three-legged structure called a triskelion. Clathrin triskelions are the assembly units of the polygonal lattice. Clathrin assembles into coats on the cytoplasmic side of the plasma membrane by interacting with its adaptor complex AP-2. AP-2 is heterotetramer consisting of two large (~100 kD) alpha and beta-2 subunits, one medium mu-2 (50 kD) and one small delta-sigma subunit (17 kD). In addition, several other proteins that play a regulatory role are found in coated pits.

The endocytic vesicles fuse with specialized membrane organelles known as endosomes which deliver the receptors and their ligands to various intracellular destinations. Internalized molecules can be either recycled back from endosomes to the plasma membrane and participate in several rounds of endocytosis, or sequestered in endosomes for a long time, or transported to lysosomes or other organelles. Here, for simplicity, we classify endosomes into early, intermediate and late endosomes according to the time of appearance of the endocytic markers in these compartments after internalization. The intravesicular pH drops along the endocytic pathway, from 6.0-6.5 in early endosomes to 4.5-5.5 in late endosomes and lysosomes, which causes dissociation of many ligand-receptor complexes (3). The mechanisms of endosomal sorting and protein coats involved in trafficking of the ligand-receptor complexes through endosomal compartments remain obscure.

3. ENDOCYTOSIS OF GROWTH FACTOR RECEPTORS: DOWN-REGULATION

For many receptors, ligand binding does not affect endocytosis and recycling, and, therefore, the level of expression. However, for receptors which transduce signals across membrane, such as receptors for polypeptide growth factors (GFs) or G protein-coupled receptors, endocytosis

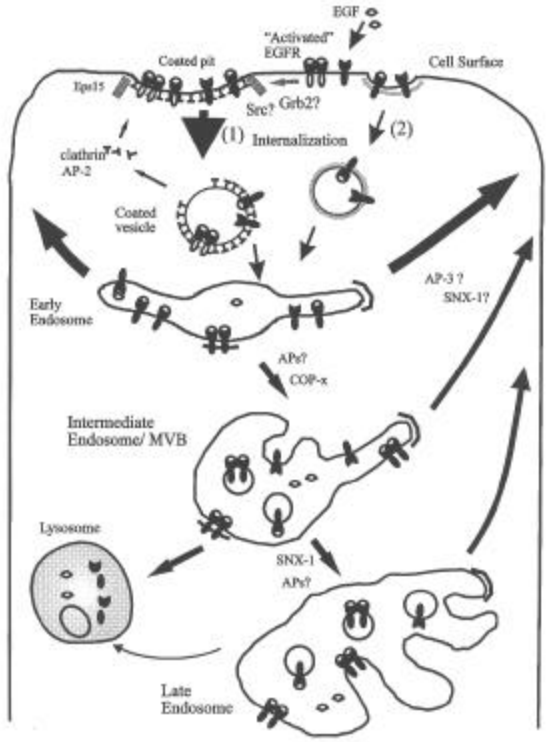

Figure 1. Pathways of internalization and intracellular sorting of GF receptors. Two pathways of endocytosis of EGF receptors are indicated: (1) high-affinity saturable pathway via coated pits (internalization rate constant k_e = 0.2-0.4, capacity is typically less than 50,000 receptors) which is utilized by EGF-activated receptors (EGFR); (2) non-saturable clathrin-independent pathway with slow kinetics (k_e=0.02-0.10) used by unoccupied and occupied EGF when the first pathway is overwhelmed. Clathrin, AP-2 and eps15 are indicated as components of the clathrin coat. The coats involved in the second pathway are not known. Both clathrin-dependent and independent internalization pathways lead receptors to the early endosomal compartments. Black arrows show recycling of occupied and unoccupied receptors from endosomes. The relative width of arrows indicates that the recycling is much slow from intermediate and late endosomes than from the early compartment. Putative endosomal sorting and coat proteins are indicated as AP-3; APs?, unidentified AP-like complexes; SNX-1 (sorting nexin-1), COP-X (coatomer (COP-I)-like proteins).

and subsequent sorting of the internalized receptors to the lysosome-degradation pathway is dramatically accelerated by the ligand. The elevated endocytosis leads to reduction of the surface and total cellular receptor pools, a phenomenon called ligand-induced "down-regulation" of the receptors.

The endocytosis of GF receptors with intrinsic tyrosine kinase activity remains the classical model system to study mechanisms of down-regulation. The specificity of down-regulation for the GF and other signaling receptors suggests that GF-induced endocytosis may play an important role in the control of GF signaling. However, the molecular mechanisms by which ligand triggers receptor endocytosis and by which endocytosis modulate signal transduction process are only poorly understood. In this review, the receptor-mediated endocytosis of receptor tyrosine kinases will be described with the focus on the mechanisms that are unique for GF receptors. A considerable amount of work has been performed using receptors for epidermal growth factor (EGF) and insulin (for review see ref. 4). We will build our discussion around endocytosis of the EGF receptor for two reasons. First, studies of this receptor have produced most of the original data on which the current model of GF receptor endocytosis is based. Secondly, insulin-induced down-regulation of the insulin receptor is typically much less dramatic than that of EGF and many other GF receptors.

4. INTERNALIZATION

4.1. Receptor recruitment into clathrin-coated pits

One group of receptors (class I) are clustered in coated pits and rapidly internalized regardless of the ligand occupancy. The well-known examples of this type are the transferrin and mannose-6-phosphate receptors. Accumulation of signaling receptors, for example EGF receptors, in coated pits is ligand-dependent (class II) (5). In the absence of EGF, EGF receptors are diffusely distributed at the cell surface (5, 6). A small pool of unoccupied receptors can be seen in coated pits (5, 7). The relative size of this pool appears to be dependent on cell type and level of receptor expression (6-8). Quantitative studies of the distribution of EGF receptors in coated and uncoated regions of the plasma membrane are difficult because the presence of receptors in coated pits is transient and its amount is very low. Several reports, however, demonstrated rapid aggregation and significant accumulation of EGF receptors in coated pits upon EGF binding (5, 6, 8-10) (figure 1).

An individual coated pit can package more than one type of receptor (11). Conformational rearrangements of the clathrin lattice result in deep invagination of the coated pit and fission of the coated endocytic vesicle containing cargo proteins. Clathrin and associated proteins must then return back to the plasma membrane to re-assemble coated pits (figure 1). Several stages of the coated pit cycle require energy, physiological temperature, and are regulated by dynamin and other GTP-binding proteins. Importantly, whereas late steps of coated vesicle formation are common for all types of receptors, receptor recruitment into coated pits appears to be the rate-limiting and ligand-dependent step of the internalization of GF receptors (10, 12).

EGF-receptor complexes can be seen in plasma membrane invaginations not covered with clathrin coat in A-431 cells (5, 13). Although the possibility exists that clathrin has dissociated during fixation and other sample preparation procedures, it has been proposed that EGF receptors can be internalized by clathrin-independent pathway in some cells. This notion is supported by the analysis of EGF endocytosis kinetics data (see below). The mechanism of clathrin-independent pathway and the nature

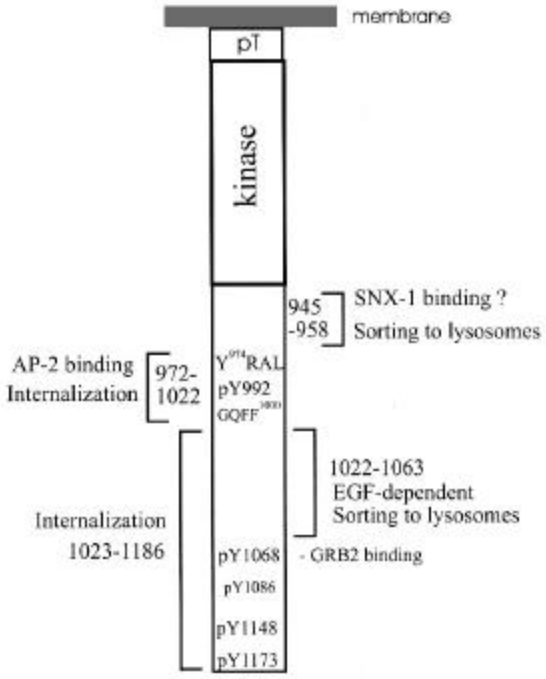

Figure 2. Trafficking signals in the cytoplasmic domain of the EGF receptor. The EGF receptor is a 170-kDa glycoprotein that is composed of three major regions: an extracellular ligand binding domain, a hydrophobic transmembrane region and a cytoplasmic domain. The extracellular and transmembrane domains are important for EGF-induced dimerization of the receptor. Cytoplasmic domain consists of conserved kinase domain located between two regulatory regions: juxtamembrane and carboxyl-terminal domains containing multiple serine, threonine and tyrosine phosphorylation sites (phosphorylated by the receptor kinase itself). Phosphorylated residues regulate receptor kinase activity and provide binding sites for other proteins. Depicted are the regions of the EGF receptor that have been implicated in the regulation of internalization and lysosomal targeting. Major tyrosine phosphorylation sites are indicated as *pY*, whereas serine and threonine phosphorylation is indicated as *pS* and *pT*. Two putative internalization motifs are Y^{974}RAL and $GGQFF^{1000}$. The first sequence has been also shown to serve as the major AP-2 binding site in the EGF receptor.

of non-clathrin coats at the plasma membrane are unknown. EGF receptors can bind to caveolae (14), which may serve as the mechanism of clathrin-independent uptake.

4.2. Molecular mechanisms of internalization

Although endocytosis of some receptors can occur in the absence of functional coated pits, clathrin-dependent endocytosis is the most efficient and fastest pathway. Receptors are recruited to coated pits efficiently and selectively because the coat proteins can recognize "endocytic codes" or "internalization signals", the sequence motifs in the cytoplasmic domains of the receptors (15). Among such signals the motifs based on a tyrosine residue and a hydrophobic residue in +3 position are the most well characterized (15). Studies with constitutively-internalized class I receptor revealed that the receptor-recognition protein in coated pits is the mu–2 subunit of AP-2 (16). Our recent studies using yeast two-hybrid system showed that EGF receptors can also bind mu-2 subunit of AP-2 (Sorkina, T, Bild, A., Tebar, F., Sorkin, A.; submitted for publication).

The EGF receptor interaction with AP-2 has been demonstrated by several *in vivo* and *in vitro* techniques (17, 18). However, it is not formally proven whether this interaction is important and sufficient for EGF-induced recruitment of receptors into coated pits. In fact, functional testing *in vivo* showed that EGFRs lacking the major AP-2 binding site can be internalized via clathrin-dependent mechanism (19, 20). Moreover, co-immunoprecipitation with AP-2 has been reported exclusively for receptors of the EGF receptor family (21, 22). The interactions of other GF receptors with AP-2 have not been shown in any type of experimental assays. Furthermore, our studies revealed that co-immunoprecipitation of EGF receptors and AP-2 is due to their interaction after internalization in endosomes (Sorkina, T, Bild, A., Tebar, F., Sorkin, A.; submitted for publication). All these data leave the question of whether the EGF receptor/AP-2 interaction is important for coated pit endocytosis, unresolved.

Several peptide sequences of the carboxyl-terminus of EGF receptor are capable of supporting rapid endocytosis and potentially binding AP-2 (23, 24). These sequences are analogous to the coated pit localization motifs (endocytic codes) found in class I receptors and contain tyrosine or phenylalanine residues (figure 2). It is not clear, however, which of these endocytic codes function in native, full-length EGF receptor, which of them are cryptic and exposed only in truncated receptor mutants, and whether different motifs can be involved in distinct pathways of internalization. Interestingly, that the tyrosine-containing motif which is essential for internalization of c-Fms receptor was found in its juxtamembrane region (25). In platelet-derived growth factor (PDGF) receptor beta, the hydrophobic region downstream of kinase domain has been implicated in the control of internalization (26). However, the kinase activity of the receptor mutants lacking this region is also severely impaired, and therefore, the direct involvement of the region in the interaction with the endocytic machinery is unlikely.

Kinetics analysis of EGF endocytosis revealed that the specific internalization rate constant of EGF internalization was several fold higher at low than at high EGF concentrations (27). Mathematical modeling of these data led to the proposal that there are two pathways of internalization of EGF receptors: a rapid saturable pathway used by a limited number of EGF-activated receptors, and a five to ten times slower non-saturable endocytosis. The latter is employed by the unoccupied receptors or by EGF-occupied receptors when the rapid pathway is saturated (12). It has been also postulated that whereas the saturable pathway involves clathrin-coated pits, the slow pathway is clathrin-independent (12).

Low capacity of high-affinity EGF-induced receptor internalization suggested that unique mechanisms may regulate this pathway. It has been hypothesized that proteins other than AP-2, expressed in limited amounts in the cell, control rapid pathway (10, 12, 28, 29). These proteins are not yet identified. The study on MDCK cells demonstrated that the microinjection of *src*-homology domains 2 (SH2) domains of GRB2 adapter protein blocks endocytosis of the EGF receptor (30). This finding is, however, difficult to reconcile with the previous results of kinetics analysis of receptor mutants (23, 24, 31, 32). For instance, mutational removal of all phosphorylation sites of the EGF receptor, including GRB2 binding, did not block EGF endocytosis (24, 32). Another putative player in EGFR internalization is the cytoplasmic tyrosine kinase c-Src that can be activated in some cell types by EGF (33).

An EGF receptor phosphorylation substrate, eps15, has been also implicated in EGF receptor endocytosis (34). Eps15 is located in plasma membrane coated pits and is associated with the -subunit of AP-2 (35, 36). However, eps15 appears to play an important role in a general endocytic machinery and is not specific for growth factor internalization (37).

Intensive studies were directed to map regions of the receptor molecule that are critical for internalization. The intracellular domain of the EGF receptor has been shown to be essential for endocytosis (figure 2). Mutational inactivation of the receptor kinase resulted in significant reduction of the internalization rate (38-42). Kinase-negative receptors were capable of internalization with a moderate speed that did not depend on EGF concentration (non-saturable pathway), suggesting that kinase activity controls the saturable pathway of internalization. Kinase activity is also necessary for the maximum rapid internalization of PDGF (43), macrophage colony-stimulating factor (m-CSF) (c-Fms receptor) (25), fibroblast growth factor (FGF) (44) and c-*kit* receptors (45). Receptor tyrosine kinases are known to be autophosphorylated but it is unlikely that kinase activity is required only for receptor autophosphorylation. For instance, an EGF receptor deletion mutant lacking all autophosphorylation sites, was internalized in kinase-dependent manner (24, 32). It is possible that tyrosine phosphorylation of an unidentified substrate of the receptor kinase is necessary for the receptor internalization through rapid saturable pathway.

Rapid internalization of EGF also requires multiple tyrosine phosphorylation of the receptor(31, 42, 46), although the role of autophosphorylation is not clear. The experiments with partially truncated receptor mutants (24) suggested that tyrosine phosphorylation *per se* is not essential for the internalization of EGF receptors. However, tyrosine phosphorylation of the full-length receptor may be needed to support conformational changes that expose endocytic codes. In PDGF receptor beta the phosphorylation site in the juxta membrane region, tyrosine 579, was found to be involved in the control of internalization (47). Phosphorylation of the c-Fms and c-*kit* receptors is not important for internalization (25, 45, 48) whereas the phosphorylation site of the FGF receptor is involved in its endocytosis (44). It is possible that observed effects of mutations of autophosphorylation sites of FGF and PDGF receptors on endocytosis reflect the necessity of phosphorylation-dependent conformational changes rather than the direct involvement of SH2-containing proteins.

In summary, rapid saturable pathway of GF endocytosis is controlled by endocytic codes, receptor kinase activity and in some cases by receptor phosphorylation. The endocytic codes presumably interact with AP-2 although other mechanisms might be involved. The nature of saturability is unknown. Receptors that lack carboxyl-terminal tail do not interact with AP-2 (49) and undergo internalization through the slow and non-saturable pathway of constitutive clathrin-independent endocytosis (figure 1).

5. INTRACELLULAR SORTING

5.1. Pathway through endosomes

After internalization, EGF and EGF receptors begin their passage through the endosomal compartments (figure 1). Clathrin-coated vesicles first fuse with early endosomes, which requires uncoating of the vesicle. Early endosomes are the tubular-vesicular compartments often connected into networks and located at the cell periphery. EGF-receptor complexes can be seen in early endosomes within 2-5 min of EGF-induced internalization at 37^0C(5, 6, 50, 51). After 10-15 min receptors begin to accumulate in large tubular-vesicular endosomes located in the perinuclear area, often surrounding the centriole (5, 6, 9, 51). On conventional electron microscopic sections, perinuclear endosomes frequently appear as multivesicular bodies (MVB) because they contain internal vesicles (5, 6, 9, 51). MVBs are likely to correspond to the intermediate and late endosomes. The transition delivery of receptors from intermediate to late endosomes/lysosomes is highly-temperature dependent as it is blocked at 16-20^{o}C (51-53). The late endosomes serve as the last destination of molecules sorted to the lysosomal pathway, and often referred to as prelysosomal compartment (54). Although the appearance of EGF receptors in late endosomes can be monitored by subcellular fractionation in density gradients, the colocalization of the EGF and mannose-6-phosphate receptors (a marker of late endosomes) has not been shown morphologically (55). Moreover, the direct fusion of MVB containing EGF receptors with lysosomes has been recently demonstrated (53). Thus, the role of late endosomes in intracellular sorting of EGF receptors is unclear.

The degradation of EGF and other GFs as well as their receptors begins after 20-30 min of continuous endocytosis (43, 56, 57), suggesting that the proteolysis occurs in MVB/late endosomes which contain functionally active enzymes. However, the complete degradation of EGF and its receptors is thought to occur in mature lysosomes. EGF and EGF receptors become detectable in lysosomes after 30-60 min of internalization but in some cells can be seen in MVBs for several hours (51). The products of partial proteolysis of the EGF receptor are

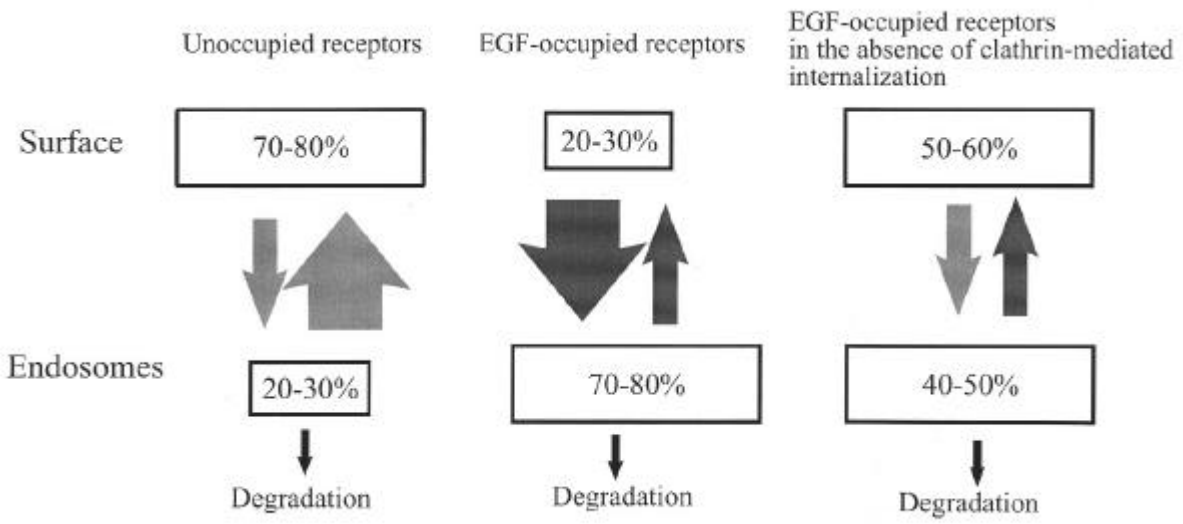

Figure 3. GF-induced changes in the rate parameters of the trafficking and subcellular distribution of the receptors. The relative values of the specific rate constants of internalization, recycling and degradation were averaged from several studies of EGF receptor endocytosis (12, 29, 31, 42, 66, 73) and expressed as the widths of arrows. The model is applicable to the cells expressing a moderate physiological amount of EGF receptors (not more than 100,000 per cell) and assumes no saturation of internalization and degradation systems. In this model we propose that EGF binding accelerates receptor internalization by activating the rapid pathway and reduces recycling of the receptors by retaining occupied receptors in endosomes and preventing them from recycling. The resulting accumulation of 70-80% of occupied receptors in endosomes after 20-30 min of continuous ligand-induced endocytosis leads to the increased apparent degradation rate and down-regulation of receptors without any change in the specific rate constant of degradation. When clathrin-dependent pathway is blocked (for instance, by expression of dominant-negative mutant of dynamin (85), EGFR down-regulation is partial.

difficult to detect in lysosomes, presumably because they are very rapidly degraded to low molecular weight peptides (58, 59).

Several lines of evidence indicate that despite an acidic environment in endosomes the release of EGF from the receptor is insignificant until the late stages of endocytosis, and that there remains a large pool of endosomal EGF-receptor complexes (60-62). This is in agreement with the common localization of EGF and EGF receptor throughout the endocytic pathway (51, 63) (figure1). Similarly, a pool of intact PDGF-receptor (64) and NGF-Trk receptor complexes (65) can be detected in endosomes. Thus, internalized GF receptors maintain their ligand-dependent dimerization and phosphorylation status during trafficking through the endosomal compartments.

5.1. Kinetics of intracellular trafficking: recycling versus degradation

The relative values of rate parameters of endocytic trafficking of occupied and unoccupied EGF receptors are compared in figure 3. In the absence of EGF, EGF receptors are slowly internalized and rapidly recycled which results in accumulation of the receptors at the cell surface (29). As described above EGF accelerates receptor internalization. EGF-receptor complexes can also rapidly recycle after internalization (41, 52, 66). The overall rate of recycling of EGF-occupied receptors is, however, 2-3 times slower than recycling of unoccupied receptors(66, 67). It can be estimated that after each round of endocytosis about 20-30% of internalized EGF-receptor complexes are trapped in endosomes and sorted to the degradation pathway, whereas only 5-10% of unoccupied receptors escape recycling. Given the low internalization rate and small pool of endocytosed receptors in the absence of EGF, the apparent degradation rate of unoccupied receptors is very low, and the loss of receptors is compensated for by biosynthesis. EGF binding reduces the recycling rate and, therefore, increases the degradation/recycling rate ratio that determines the fate of receptor population after endocytosis. Reduced recycling together with EGF-accelerated internalization result in the significant elevation of the overall degradation of receptors which leads to receptor down-regulation.

The importance of ligand binding for the lysosomal targeting and down-regulation of internalized receptors can be illustrated by the example of the differential effect of EGF and transforming growth factor alpha (TGFalpha) on endocytosis of their common EGF receptor. TGFalpha binding to the EGF receptor is much more pH-sensitive than binding of EGF to the receptor and, probably, most of TGFalpha dissociates from the receptor at pH 5.5-6.0 in early and intermediate endosomes (68, 69). This dissociation allows rapid recycling of ligand-free receptors and reduces overall receptor degradation. Consequenly, TGFalpha causes much weaker down-regulating effect on EGF receptors compared to that of EGF (68, 69).

5.3. Possible mechanisms of receptor sorting in endosomes

Recycling of EGF-receptor complexes is partially inhibited at 18°C when receptors are accumulated in MVB/intermediate endosomes, suggesting that recycling does occur from these compartments (52). This "late" recycling pathway is, however, much slower than recycling from early endosomes. In general, the deeper receptors advance along the endocytic pathway, the slower the overall rate and the smaller the extent of receptor recycling. The reduced recycling correlates with elevated sorting to the degradation pathway. To understand the mechanisms of the retention of EGF-receptor complexes in "maturing" endosomes during endocytosis, the pathways of intracellular sorting of EGF receptors have been compared with those of transferrin receptors which are targeted to lysosomes very insignificantly (9, 50). Morphological analysis revealed that in early endosomes and MVB EGF receptors tend to accumulate in vesicular parts of the compartments (figure 1), whereas transferrin receptors are mostly located in tubular parts (9). Moreover, in MVB-like endosomes, EGF receptors are preferentially associated with the internal vesicular structures, whereas transferrin receptors are distributed mainly in the outer membrane and tubular parts of endosomes. On the basis of these observations, the following model of endosomal sorting has been proposed (9, 70). Receptors located in the tubular portions of endosomes are constitutively recycled. In contrast, a pool of EGF receptors are trapped (retained) in the vesicular parts and subsequently become incorporated into internal vesicles of MVBs. According to this model, receptor packaging into internal vesicles prevents their recycling and leads to their retention in MVB/late endosomes and subsequent exposure to proteolytic enzymes after endosome-lysosome fusion.

The molecular mechanisms of the selective sorting of occupied EGF receptors to the internal membranes of endosomes are unknown. It has been hypothesized that EGF-induced activation of receptor kinase and tyrosine phosphorylation of annexin I in MVBs is important for inclusion of EGF receptors in internal vesicles of MVBs (71, 72). However, the data that receptor mutants lacking an ATP-binding site or an entire kinase domain are degraded as fast as wild-type receptors, have questioned the direct involvement of tyrosine kinase activity in the intracellular sorting of EGF receptors (23, 29, 66, 73). Recent reports (23, 74) suggested that carboxyl-terminal regions of the receptor between residues 945-958 and 1022-1063 (figure 2) are necessary for the receptor degradation. Interestingly, kinetic studies revealed that the lysosomal sorting (or retention) pathway of EGF receptors is also saturable (73), indicating that either the proteins responsible for sorting or the pool of EGF receptors competent for lysosomal sorting are limited. Whereas degradation of large concentration of endosomal EGF receptors did not depend on the receptor kinase activity, the saturable pathway is apparently controlled by the receptor kinase (74).

An endosomal protein called sorting nexin, SNX-1 has been recently discovered based on its ability to interact with the cytoplasmic domain of the EGF receptor in yeast two-hybrid system (75). Overexpression of SNX-1 leads to down-regulation of the EGF receptor but not other GF receptors suggesting that SNX-1 plays an important role in the specific targeting of EGF receptors to the degradation pathway. PDGF-dependent activation of phosphatidylinositol-3-kinase has been implicated in regulation of the endosomal sorting of the PDGF receptor (76). However, this kinase appears to play role in some universal steps of endosomal sorting and is not specific for GF endocytosis. The protein coats and adaptor molecules involved in endosomal sorting of GF receptors are unknown. Recently, clathrin coats containing adaptor complex AP-3 have been identified in the early endosomes of A-431 cells (77). It is attractive to propose that these coats are involved in recycling of receptors as they contain transferrin receptors (78).

6. ROLE OF ENDOCYTOSIS IN GROWTH FACTOR SIGNALING

GF-induced receptor down-regulation is thought to serve as feedback attenuation of mitogenic and transforming receptor signaling (79). The main argument to support this notion is the observation of an elevated mitogenic, transforming and tumorogenic activity of the EGF receptor mutant that is internalized very inefficiently (80). In physiological systems endocytosis can negatively regulate signaling by at least two ways. First, ligand-induced down-regulation leads to significant reduction of the total receptor pool in the cell. Second, extensive endocytosis may lead to depletion of GF from the extracellular fluid (79). The saturability of the specific endocytic pathway of internalization and lysosomal sorting has important implication in respect of regulation of signaling by endocytosis. Cells that express moderate levels of GF receptors can balance GF signaling by down-regulating GF receptors. In contrast, cells that overexpress GF receptors are incapable of efficient down-regulation of these receptors because the endocytic machinery is overwhelmed and can not handle the large amount of activated GF receptors. In results, a large pool of active GF receptors is maintained in such cells for the long periods of time in the presence of GF, which leads to extensive and unbalanced GF signaling. An example of such system are several types of breast and squarmous carcinoma cells that overexpress EGF receptors (81).

Another conceivable consequence of endocytosis for GF signaling is the possibility of signaling from endosomes. Studies on perfused rat liver and tissue culture cells demonstrated that EGF remains bound to the receptor during endocytosis, and that receptors remain dimerized, tyrosine phosphorylated, active as kinase and associated with the signal transduction proteins in endosomes (61, 62, 82, 83). Similarly, the existence of active PDGF receptors β in endosomes has been proposed (64). In neurons, there is a physical distance between the site of initial activation of Trk receptor tyrosine kinase by the nerve growth factor (NGF) in the nerve terminal and the site of action in the cell body. It has been proposed that activated Trk is transported to the cell body in specialized endosomes where it preserves kinase activity (65). Thus, "signaling" endosomes may be important in

mediating a variety of early and late signaling events in GF-stimulated cells.

An important limitation of the signaling from endosomes is the sequestration of receptors into internal vesicles of MVB/intermediate/late endosomes. While the carboxyl-terminal domain of the receptors located in the limited endosomal membrane is accessible to kinase substrates and stably interacting proteins, most receptors are accumulated in the internal vesicles within several minutes of continuous endocytosis and cannot be involved in signaling. Whether internal vesicles are physically separated from the limited membrane of endosomes is not clear. Large perinuclear endosomes appear on frozen sections as the tubular-vesicular structures of complex morphology rather than round-shaped MVB seen on the conventional Epon sections (51). It can be proposed that at least some of the vesicle profiles are the deep invaginations of the limited membrane of the endosome.

Despite the possibility of signaling from endosomes, the existence of endosome-specific signals is yet to be demonstrated. The importance of endocytosis for activation of the individual signal transduction pathways has been demonstrated in HeLa cells in which clathrin-dependent endocytosis of EGF receptors was inhibited by overexpression of the dominant-negative mutant dynamin (84). The EGF-induced DNA synthesis was not affected in the absence of endocytosis whereas the expression of mutant dynamin abolished the MAP (ERK) kinase activation by EGF. How receptor-mediated endocytosis controls MAP-kinase activation remains to be determined.

7. PERSPECTIVES

We have discussed pathways, kinetics, possible molecular mechanisms and role in signaling machinery of the receptor-mediated endocytosis of GFs. Although several molecules that interact with EGF receptors and might be involved in endocytic trafficking of these receptors have emerged, the mechanism and the role of these interactions needs further investigation. It is important to determine whether the same or similar proteins control endocytosis of other GF receptors. Certainly, new components of endocytic machineries that are specific for signaling receptors will be discovered. Heterologous expression of sorting proteins may allow the manipulation of the levels and activities of GF receptors in cancerous cells. On the other hand, sequence motifs within GF receptors that are important for their trafficking should be mapped to allow structure-function analysis of receptor interaction with the sorting proteins. Finally, we predict that the issue of relationships between endocytosis and cell signaling will be in a focus of future investigation.

8. ACKNOWLEDGEMENTS

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