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Review

Regulation of EGFR endocytic trafficking by rab proteins

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Summary. The Epidermal Growth Factor Receptor (EGFR) is a member of the receptor tyrosine kinase family and has important roles in development and cancer. Through ligand stimulation, the EGFR initiates a number of biochemical pathways that integrate to form specific physiological responses. In addition to these signaling pathways, the ligand stimulation also causes the EGFR to internalize and be transported through the endocytic pathway. The endocytic pathway regulates the rate of EGFR degradation and recycling, as well as the signaling mediated by the EGFR. In this review, the role of rabs, a family of small molecular weight guanine nucleotide binding proteins, is examined in how they regulate endocytic trafficking.

Key words: Rab, Growth factor receptor, Trafficking, EGFR

Introduction

The Epidermal Growth Factor Receptor (EGFR) is a member of the receptor tyrosine kinase (RTK) family that plays an important role in cell growth and development (Jorissen et al., 2003). The EGFR is a single membrane spanning protein with an extracellular amino terminus and an intracellular carboxyl terminus. Ligand binding to the extracellular domain induces a conformational change in the receptor that promotes dimerization and subsequent activation of the receptor's intrinsic tyrosine kinase domain. Through transphosphorylation, the kinase domain of one receptor phosphorylates tyrosine residues on the carboxyl terminus of its partner. These phosphotyrosines serve as docking sites for downstream signaling molecules – either enzymes such as phosphatidyl inositol 3-kinase (PI3K), phospholipase C gamma (PLCγ), Src, and c-Cbl or adapator proteins like SHC and Grb2 that serve as intermediates for other signaling events (Olayioye et al., 2000; Yarden and Sliwkowski, 2001).

Activation of the EGFR results in a plethora of physiological consequences including embryonic development, cell growth, and cell differentiation (Jorissen et al., 2003). In addition, the EGFR is overexpressed and/or hyperactivated in cancers of diverse anatomic origin including breast, ovary, renal, non-small cell lung carcinoma, head and neck cancers, colorectal, and pancreatic (Huang et al., 1997; Rowinsky, 2004). Thus, the EGFR is a protein of great interest to both developmental and cancer biologists.

There are six naturally occurring ligands that can activate the EGFR (Holbro and Hynes, 2004). The regulated release of these ligands determines when the receptor becomes activated. While ligand binding is clearly the rate-limiting step in the initiation of receptor signaling, there are multiple processes that contribute to the inactivation of receptor signaling, such as receptor dephosphorylation, receptor degradation, and effector/substrate depletion. Much of the attention regarding receptor inactivation has focused on ligand-stimulated, endocytic trafficking (Wiley, 2003).

In the resting state, most EGFRs reside on the cell surface while a basal level of receptors are internalized by endocytosis (Herbst et al., 1994). Upon stimulation with ligand, internalization of activated receptors through clathrin-coated pits is enhanced. Inside the cell, the newly formed clathrin-coated vesicles, shed their clathrin and become intermediate vesicles that fuse to the early endosome. In the early endosome, cargo is sorted and readied for trafficking to its final cellular destination. Depending on the cell line, EGFRs may be trafficked to the late endosome and then onto the lysosome for degradation, the endoplasmic reticulum and golgi for reprocessing, or recycled to the plasma membrane. When the receptor is targeted for degradation, the EGF/EGFR complex becomes internalized within the endosome to form what is called the multivesicular body (MVB) (Stahl and Barbieri, 2002). Delivery of this cargo from the late endosome to

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the lysosome is mediated by a temporary fusion of the two compartments, transfer of the contents to the lysosome, and re-separation into the original parent compartments. This is referred to as the "kiss and run" model (Wiley, 2003) (Fig. 1).

Historically, trafficking through the endocytic pathway has been thought of as a mechanism to "turn off" or inactivate the stimulated receptor, however, more recently it has been recognized as a mechanism for regulating signaling to downstream effectors (Ceresa and Schmid, 2000; Leof, 2000). This paradigm shift underscores the importance of understanding the molecules and mechanisms that regulate the movement of the EGFR in order to reveal important insights into basic cell biology and illuminate the pathophysiological role of EGFR endocytosis. For example, newly identified proteins can be expressed as mutants or knocked-down to alter EGFR trafficking and allow one to assess the signaling contribution of the EGFR when enriched at a specific endocytic stage.

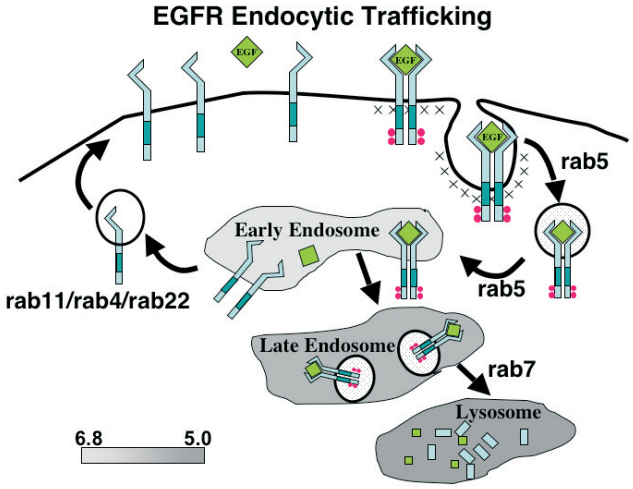

Fig. 1. Schematic of EGFR Endocytic Trafficking. In the liganded state, the EGFR is a monomer on the cell surface. Ligand induces the dimerization of the EGFR, activates the intracellular kinase domain, and transphosphorylation of intracellular carboxy-terminal tyrosine residues that serve as docking sites for downstream signaling proteins. The ligand-bound receptor translocates to domains of the plasma membrane with an intracellular clathrin lattice. The clathrin lattice invaginates to form a clathrin-coated pit that pinches off to form a clathrin-coated vesicle. Once the clathrin is shed from the clathrin-coated vesicle, this intermediate vesicle fuses with the early endosome, delivering the receptor. In the early endosome, the receptor is readied for its appropriate cellular fate – recycled back to the plasma membrane, trafficked to the lysosome for degradation, or targeted to another subcellular compartment. For those receptors that are ultimately degraded, another series of budding/fusion reactions transport the receptor to the late endosome. Receptors enter multivesicular bodies that form from invagination of the endosomal membranes. Receptors are transferred from the late endosome to the lysosome when the two organelles temporarily fuse, exchange cargo, and then separate into their original compartments ("kiss and run"). The pH gradually decreases in the progression from the plasma membrane to the lysosome (indicated by increased shading).

Rab proteins

To fully understand the molecular regulation of EGFR signaling, recent efforts have focused on identifying the proteins that regulate the endocytic trafficking of the receptor. This review will focus on the role of a class of small molecular weight guanine nucleotide binding proteins (rabs) that regulate the movement of the activated EGFR within the cell.

Rabs are a family of over 60 proteins that are defined by their similar structure, function, and guanine nucleotide binding properties (Pfeffer and Aivazian, 2004; Seabra and Wasmeier, 2004). Like all GTPases, rabs can bind guanosine triphosphate (GTP) and guanosine diphosphate (GDP), and the nucleotide bound determines the activation state of the enzyme (Fig. 2). Binding of GTP activates rabs whereas GDP binding inactivates them. The exchange between these two states is regulated by guanine nucleotide exchange factors (GEFs) that initiate the dissociation of GDP. Due to higher cytosolic levels of GTP, the guanine nucleotide binding pocket will preferentially be occupied by GTP. The conversion from an active to an inactive form is mediated by stimulation of a GTPase activing protein (GAP) that activates the intrinsic GTPase domain of the rab causing the hydrolysis of the gamma phosphate from GTP, resulting in a GDP bound form of rab and the liberation of inorganic phosphate (Pi). The GTP-bound

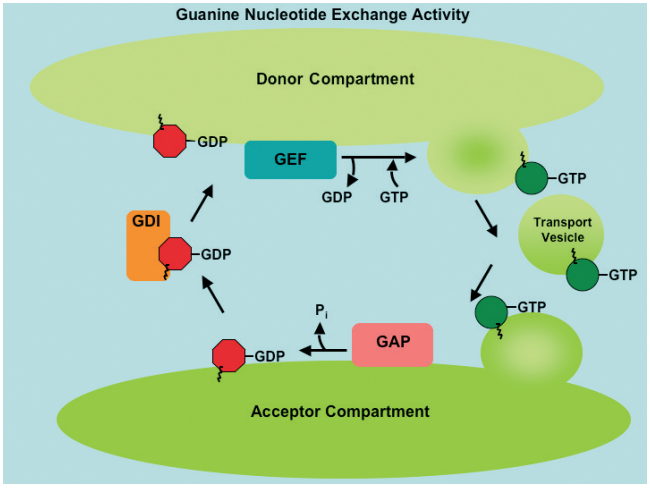

Fig. 2. Schematic of rab proteins in vesicular transport. Rab proteins bound to GDP is anchored to the donor membrane via a lipid group. Stimulation by the guanine nucleotide exchange factor (GEF) leads to dissociation of the bound GDP which is replaced by GTP due to higher cellular concentrations. The GTP-bound rab associates with the budding vesicle and is transported to the acceptor compartment and fuses with the membrane. The gamma phosphate of the bound rab-GTP is hydrolyzed (Pi) by the rab's intrinsic GTPase activity that is stimulated by a GTPase activating protein (GAP). This process inactivates the rab and readies it for recycling back to the donor membrane. The guanine nucleotide dissociation inhibitor (GDI) transports the inactive rab from the acceptor membrane to the donor membrane.

active rab transitions from donor to acceptor membrane via the target vesicle and returns to the donor membrane by a Guanine nucleotide Dissociation Inhibitor (GDI) (van der Bliek, 2005).

As a whole, the rab family of proteins are responsible for mediating vesicular trafficking events by regulating the formation of new vesicles (budding) and the fusion of vesicles. Despite a high level of structural, molecular, and mechanistic similarity, these proteins are very specific in the cellular functions that they mediate. This specificity is conferred by a) the subcellular localization of the rab and b) the upstream and downstream effector proteins. Thus, each rab protein is able to mediate a specific membrane trafficking event in response to a given activator.

To date, only a subset of rab proteins have been shown to regulate EGFR endocytic trafficking. Much of the information regarding the biological role of rabs has been generated through modulation of the GTPase activity and guanine nucleotide binding pocket. Mutation of a key glutamine (often to a leucine) in the GTPase domain of rabs results in a protein with an inactive GTPase domain that is constitutively loaded with GTP (Dumas et al., 1999). The generation of dominant negative rabs can be made by mutating the guanine-nucleotide binding pocket such that it does not bind any nucleotide or preferentially binds GDP (Dumas et al., 1999). By overexpressing these mutant proteins, the normal rab-interacting machinery can be perturbed and the resultant phenotypes can reveal what stages in endocytic trafficking each rab regulates as well as the consequence on the endocytic trafficking of the EGFR.

This discussion of rab proteins in EGFR endocytic trafficking has been divided into three parts: receptor endocytosis, receptor recycling, and receptor degradation.

Rabs in EGFR endocytosis

Rab5

Rab5 is the best-studied rab protein regarding EGFR trafficking. First cloned from Madin Darby Canine Kidney (MDCK) cells by Chavrier et al. (1990), it is now appreciated that there are three human rab5 isoforms – rab5a, rab5b, and rab5c (Bucci et al., 1995). At the cellular level, rab5 is localized to both the plasma membrane and the early endosome (Chavrier et al.,

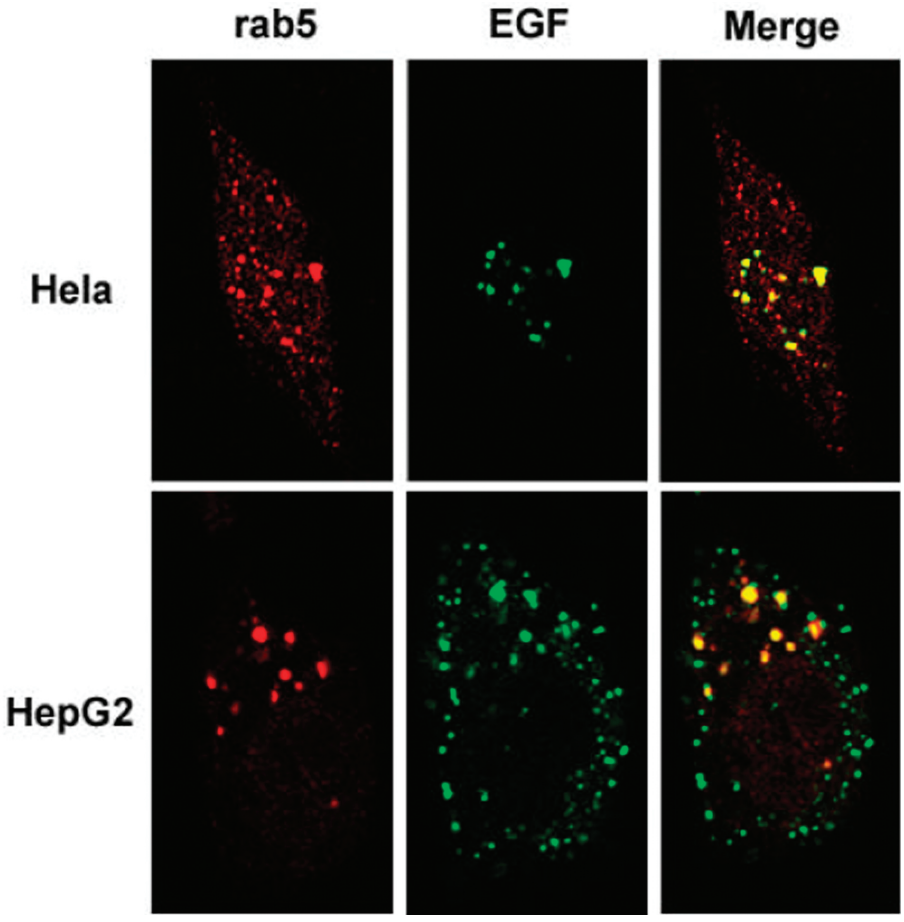

Fig. 3. Endogenous EGFRs localize with endogenous rab5. Uninfected HeLa and HepG2 cells were incubated with Alexa488-EGF ('EGF' – green) for 10 minutes at 37°C. Cells were fixed, and processed for immunofluorescence with an anti-rab5 antibody ('rab5' – red) (Transduction Labs). Merge shows the co-localization of the internalized EGF:EGFR complex with the endogenous rab5. Cells were visualized with Leica NTS confocal microscope. (100X objective).

1990), and a role for rab5 in the biogenesis of the early endosome is well established (Gorvel et al., 1991; Bucci et al., 1992). Expression of activated mutants of rab5 (rab5Q79L) results in the formation of an enlarged early endosome (Stenmark et al., 1994), as indicated by the presence of the marker protein, early endosomal antigen 1 (EEA1) (Dinneen and Ceresa, 2004a; Mu et al., 1995).

There are several reports describing a role for rab5 in regulating endocytic trafficking of the EGFR, however, a question remains as to exactly which point in the endocytic pathway is being regulated. Evidence exists supporting a role for rab5 regulating EGFR trafficking at the plasma membrane (Barbieri et al., 2000; Huang et al., 2004) and at the early endosome (Dinneen and Ceresa, 2004a).

It has been shown by numerous groups that stimulated EGFR accumulates in rab5-positive early endosomes (Mu et al., 1995; Roberts et al., 1999; Barbieri et al., 2000; Chen and Wang, 2001; Dinneen and Ceresa, 2004a) (Fig. 3). The question that remains is whether rab5 is directly responsible for regulating the entry of the EGFR into this compartment. Prolonged expression of activated rab5 in HeLa cells causes the accumulation of unliganded EGFR in the enlarged endosome (Dinneen and Ceresa, 2004b). This is clearly the consequence of redistribution of cell surface EGFRs as the increase in endosomal EGFRs is accompanied by a corresponding decrease in cell surface EGFR. In addition, marking the cell surface EGFR with an antagonistic antibody results in the accumulation of receptors in the endosome (Dinneen and Ceresa, 2004b). However, it remains to be determined whether the changes in EGFR distribution are the result of increased endocytosis or an inhibition of internalized receptor recycling.

The role of rab5 in ligand-mediated EGFR endocytosis has been studied by multiple groups. Barbieri et al. used a radioligand internalization assay to show that expression of dominant negative rab5a results in a greater than 50% decrease in EGFR internalization in NR6 mouse fibroblasts, whereas overexpression of wild type rab5a enhances EGFR endocytosis (Barbieri et al., 2000). A similar inhibition in EGFR endocytosis was seen with expression of dominant negative rab5a in porcine aoric endothelial cells expressing EGFR (PAE/EGFR) using a fluorescent assay (Galperin and Sorkin, 2003).

Experiments by Dinneen and Ceresa expressing active and inactive mutants of rab5 showed no change in the rate of EGFR internalization in HeLa or HepG2 cells (Dinneen and Ceresa, 2004a). However, downstream endocytic effects were observed in cells expressing rab5 (S34N), a mutant that preferentially binds GDP. Specifically, rab5 (S34N) slowed the rate of ligand-stimulated EGFR degradation and failed to co-localize with the internalized EGF/EGFR. These data are consistent with rab5 (S34N) disrupting formation of the early endosome (Dinneen and Ceresa, 2004a).

Work from the Sorkin lab provides additional support that rab5 may be working at the plasma membrane (Huang et al., 2004). Using a transient RNAi knockdown strategy, this group demonstrated that when all three isoforms of rab5 are eliminated, there is a 50% decrease in the amount of EGFR internalized in HeLa cells. The single knockdown of any isoform alone did not cause greater than a 10% reduction in EGFR endocytosis, suggesting a functional redundancy between the isoforms.

The challenge is to interpret the data generated from these disparate assays. One possibility is that the dominant negative form of human rab5a may be sufficient to inhibit the function of all rab5 isoforms, whereas its canine counterpart cannot. Although the human and canine sequences only differ by two amino acids, these two differences may have functional consequences. This hypothesis might be readily tested by analyzing chimeric rab5 proteins. Alternatively, there may be a different complement of rab5 isoforms expressed in various cell lines, thereby being functionally redundant in only some cells. Finally, we must consider the differences in EGFR expression in the cell lines. The cell lines used to demonstrate a role for rab5 at the cell surface had exogenously expressed EGFRs, whereas when rab5 was shown to regulate trafficking to the early endosome the cells contained endogenous EGFR.

Additional evidence supporting a role for rab5 in EGFR trafficking comes from studies with the rab5 guanine nucleotide exchange factor (GEF), rin-1. Tall et al. found that rin-1 associates with the EGFR in a ligand-dependent manner and overexpression of rin-1 increases the basal level of EGFR endocytosis, presumably through enhancing rab 5 activation (Tall et al., 2001). Interestingly, expression of rin-1 increases rab5 activation but does not produce the enlarged endosomes that are characteristic of the constitutively GTP-loaded mutant of rab5 (Tall et al., 2001; Barbieri et al., 2003, 2004). One explanation may be that excess rin-1 may still allow cycling of rab5 between GTP- and GDP-bound states, whereas the activating mutant of rab5, does not leading to an enlarged endosome. Nevertheless, rin-1 provides a biochemical link between cell surface and endosomally-localized EGFR.

Bearing in mind the different cell lines, assays, and mutant rab5 proteins used in the abovementioned studies, the data obtained help us model how rab5 functions in EGFR trafficking. Rab5 likely functions at both the plasma membrane (donor membrane) and the early endosome (acceptor membrane) as suggested in the model in Figure 2. Identifying these multiple sites of action have only been elucidated by varying approaches from individual labs.

Rab11

There is evidence that a second rab, rab11, regulates EGFR endocytosis. Rab11 has been shown to localize to the recycling endosome, so its role in receptor

internalization is unexpected. Although overexpression of GFP-tagged rab11 has no effect on EGFR endocytosis (Barbieri et al., 2000), expression of a rab11 interacting protein does. Rab11-FIP2 was identified from a yeast two-hybrid screen using rab11 as bait and binds both active and inactive rab11 (Hales et al., 2001). Transfection of COS-7 cells with rab11-FIP2 inhibits the endocytosis of EGF, but not transferrin, indicating its selectivity in cargo (Cullis et al., 2002). As pointed out by the authors of this work, it remains unclear if rab11-FIP2 has a physiological role in EGFR endocytosis. It may well be that the normal function of rab11-FIP2 is to facilitate recycling but that over-expression of the protein inhibits endocytosis by interfering/disrupting with key components of the endocytic machinery. Further studies are clearly needed to resolve the true function of this rab11 effector. It may be that rab11-FIP2 interacts with both the internalization machinery and rab11. Rab11-FIP2 may function in EGFR internalization and then interact with rab11 to be returned to the plasma membrane.

RABs in EGFR recycling

Once the EGFR enters the early endosome, the receptor either continues down a degradation pathway or is recycled back to the plasma membrane (Fig. 1). Due to differences in the extent of EGFR recycling in various cell lines, the physiological relevance of this process is poorly understood. The extent of EGFR recycling can vary widely, as 20-80% of activated EGFR may be targeted to the lysosome in a given cell line (French et al., 1994; Worthylake et al., 1999). Since the lysosomal trafficking of the EGFR is saturable (French et al., 1994), many of the differences in the extent of EGFR recycling reported may be attributable to either over-expression of the receptor or to treatments that disrupt the endocytic machinery. Further, the extent of receptor recycling can be altered by ligand. Due to pH sensitivity, EGFRs stimulated with transforming growth factor α (TGF α) preferentially recycle as compared to those that are stimulated with EGF (Waterman et al., 1998). In addition, the presence of EGFR family members (i.e. ErbB2, ErbB3 and ErbB4) can promote EGFR recycling when ligand-stimulated, heterodimeric complexes are formed (Waterman et al., 1998). These issues add an additional level of complexity to assessing the role of rabs in EGFR recycling.

Only three rab proteins have been implicated to have a role in EGFR recycling. Mentioned above was the role of rab11; in addition, rab4 and rab22a have been studied.

Rab4

Human rab4 was cloned from a pheochromocytoma cDNA library that was screened with a rat rab4 probe (Zahraoui et al., 1989). Initial studies using biochemical fractionation approaches described rab4 as localizing to the early endosome and being involved in regulating the recycling of the transferrin receptor (van der Sluijs et al., 1991). Despite the existence of this data for the past 15 years, relatively little is known about rab4's role in regulating EGFR trafficking. According to models that have been put forth by Zerial et al., rab5 regulates trafficking into the cell by regulating fusion of intermediate vesicles with a specific domain of the early endosome whereas rab4 regulates budding of a separate domain of the same endocytic vesicle (Sonnichsen et al., 2000).

Studies in HeLa cells examining the role of rab4 in EGF degradation have revealed that expression of a mutant that preferentially binds GDP (rab4S22N) has a 30% reduction in EGF degradation. However, the corresponding activating mutant of rab4 did not have the reciprocal effect on EGF degradation (McCaffrey et al., 2001). Once again, the question remains as to whether rab4 directly affects EGFR degradation, or whether expression of the mutant inhibits endosomal fluidity by disrupting the machinery for endosomal recycling.

Rab22a

Rab22a is localized to the early endosome, interacts with EEA1, and shares a high level of homology to rab5a (Chen et al., 1996). Functionally, it appears to regulate receptor recycling rather than internalization. Expression of an activated mutant of rab22a results in the formation of an enlarged endosome similar to that caused by the active mutant of rab5 (Kauppi et al., 2002). Unlike the rab5 mutants, overexpression of wild type, activating, or inactivating mutants of rab22a in HepG2 cells decreased the degradation of fluorescently labeled-EGF as measured by the formation of EGF containing endosomes (Kauppi et al., 2002). In addition, expression of wild type and mutant rab22a caused the redistribution of various endocytic marker proteins.

Regarding the EGFR, these data provide important information concerning endocytic trafficking. Since activating and inactivating mutants yield the same consequence, this is consistent with a requirement for the constant recycling of rab22a and may implicate multiple roles for this rab. Two plausible explanations are that GDP- and GTP-bound rab22a interact with different proteins, each of which is important for EGFR trafficking. Alternatively, like rab11, there may be an effector that binds to both the activated and inactivated forms of rab22a and overexpression of either mutant may sequester necessary proteins for trafficking.

Rabs regulating EGFR degradation

Among the rab proteins shown to be localized to the late endosome are rab7, rab9, and rab34. To date, rab9 and rab34 have not been reported to play a role in regulating EGFR trafficking, which is not surprising based on their role in regulating golgi-lysosomal transport (Lombardi et al., 1993; Speight and Silverman, 2005). However, there is growing evidence that rab7

functions in the regulated degradation of the EGFR.

Rab7 was initially cloned from MDCK cells and shown to be localized to the late endosome (Chavrier et al., 1990). Early descriptions of rab7 function clearly indicated a role in endocytic trafficking, however, it was unclear as to whether the small GTPase was regulating the flow of cargo into or out of the late endosome (Feng et al., 1995; Mukhopadhyay et al., 1997; Press et al., 1998; Bucci et al., 2000).

Studies performed in HeLa cells demonstrated that overexpression of dominant negative rab7 slowed the rate of both EGF and EGFR degradation whereas activating mutants of rab7 accelerated degradation of the EGF/EGFR complex. Importantly, when dominant negative rab7 is used to slow the rate of EGF/EGFR degradation, the complex accumulates in the late endosome. Taken together, these data support a role for rab7 in regulating EGFR endocytic trafficking out of the late endosome (Ceresa and Bahr, 2006).

Two rab7 interacting proteins have been indentified: XAPC7 (a proteasome α-subunit) and RILP (Rab-interacting lysosomal protein) were identified in independent yeast two hybrid screens using the GTP-bound form of rab7 as bait (Cantalupo et al., 2001; Dong et al., 2004). Overexpression of either protein causes an inhibition of EGF/EGFR trafficking as measured by fluorescently labeled EGF and radioligand degradation (Cantalupo et al., 2001; Dong et al., 2004). In addition to supporting the role of rab7 in EGFR degradation, these data provide evidence of a stoichiometric balance between rab7 and its effectors that is necessary for the regulated degradation of the EGFR.

Conclusions

The studies discussed in this review highlight the importance of rab proteins in the directed endocytic trafficking of the EGFR. However, there are several issues that must be kept in mind. First, many of these studies have relied on the overexpression of activating and inactivating mutants of the individual rabs. Therefore, there is the caveat that the observed phenotype reflects the sequestration of upstream proteins rather than the true site of rab function. Second, these data by-in-large overlook the issue of specificity in endocytic transport as many of these rab protein have been shown to regulate the trafficking of other signaling receptors. This raises the question: What confers specificity to EGFR trafficking? One possibility is that the EGFR mediates guanine nucleotide exchange on a subset of rab family members. Alternatively, there may be receptor specific adaptor proteins that guide the EGFR through the various endocytic compartments. Nevertheless, a partial map of the rabs involved in EGFR trafficking has been assembled. While this map is certainly incomplete, it lays the framework for understanding the role of endocytic trafficking in EGFR function.

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