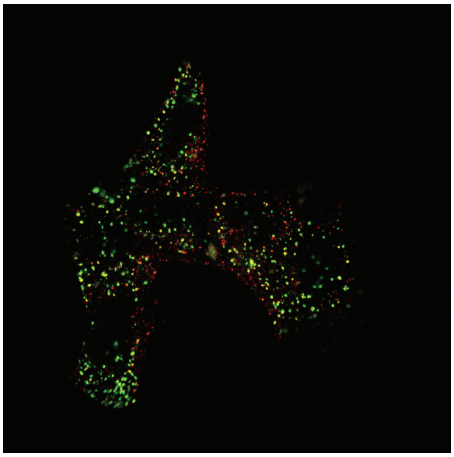
Proteins and mechanisms involved in endosomal sorting of the epidermal growth factor receptor

Lene Melsæther Grøvdal



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Oslo, January 2007
Lene M. Grøvdal

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II. Myromslien, F.D., **Grøvdal, L.M**., Raiborg, C., Stenmark, H., Madshus, I.H., and Stang, E. 2006. Both clathrin-positive and -negative coats are involved in endosomal sorting of the EGF receptor. *Exp Cell Res.* 312. 3036-3048.

III. **Grøvdal, L.M**., Johannessen, L.E., Rødland, M.S., Madshus, I.H., and Stang, E. Over-expression of Ack1 inhibits internalization and endosomal sorting of the EGF receptor. Manuscript.

ABBREVIATIONS

AAK1	Adaptor-associated kinase
Ack	Activated Cdc42 associated kinase
AP	Adaptor protein complex
AR	Amphiregulin
ARH	Autosomal recessive hypercholesterolemia
CALM	Clathrin assembly lymphoid myeloid leukemia protein
CHC	Clathrin heavy chain
CLC	Clathrin light chain
CR	Cysteine rich
Dab2	Disabled-2
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EH	Eps15 homology
EM	Electron microscopy
ENTH	Epsin amino terminal homology
EPR	Epiregulin
Eps15	EGFR-pathway substrate-15
ESCRT	Endosomal sorting complex required for transport
FYVE	Fab1, YOTB/ZK632.12, Vac1 and EEA1
GAK	cyclin-G associated protein kinase
GED	GTPase effector domain
GEF	Guanine nucleotide exchange factors
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
HB-EGF	Heparin-binding EGF
HECT	Homologous to E6AP carboxy terminus
Hrs	Hepatocyte growth factor regulated tyrosine kinase substrate
L	Large domain
LDLR	Low-density lipoprotein receptor
MHC-I	Major histocompatibility complex class I
MVB	Multivesicular bodies
N-WASP	Neural Wiscott-Aldrich syndrome protein
PH	Pleckstrin homology
PI	Phosphatidylinositol
PIP	Phosphatidylinositol phosphate
PI(3)K	Phosphatidylinositol 3-kinase
PRD	Proline rich domain
PTB	Phosphotyrosine binding
pY	Phosphorylated tyrosine
RING	Really interesting new gene
SH	Src homology
STAM	Signal-transducing adaptor molecule
TGF-α	Transforming growth factor alpha
TfR	Transferrin receptor
TGN	*trans*-Golgi network
TKB	Tyrosine kinase binding
UBA	Ubiquitin-assosiated
UIM	Ubiquitin-interacting motif
VHS	Vps27, Hrs and STAM
Vps	Vacuolar protein sorting
WASP	Wiscott-Aldrich syndrome protein
wt	Wild type

INTRODUCTION

Cellular growth and migration is regulated by growth factors. Extracellular growth factors bind to transmembrane receptor proteins. Binding of growth factors to the extracellular domain of the receptors causes activation of the receptor and starts signaling cascades inside the cell, initially by the recruitment of other proteins to the intracellular domain. One of the first growth factor receptors to be identified was the Epidermal Growth Factor Receptor (EGFR) (Cohen, 1962). This receptor is activated by binding a specific set of growth factors, and as a response it binds and phosphorylates other proteins inside the cell. This leads to the onset of signaling cascades which are again terminated by internalization and deactivation of the receptor.

Structure and activation of the EGFR

The EGFR is a member of the EGFR family of receptor tyrosine kinases, which in addition to the EGFR (ErbB1) consists of ErbB2, ErbB3 and ErbB4. These receptors are widely expressed in human tissues and are involved in processes like development, proliferation and differentiation (reviewed in Olayioye *et al.*, 2000). Extracellularly, the EGFR contains a 620 aa ligand binding domain consisting of four domains (I-IV) (Lax *et al.*, 1988) (see Figure 1). The domains also known as the two large (L) domains and the two cysteine-rich (CR) domains are important for ligand binding and dimerization (Lax *et al.*, 1989; Garrett *et al.*, 2002; Ogiso *et al.*, 2002). The transmembrane domain spans the plasma membrane, supposedly as an α-helix (Rigby *et al.*, 1998) and connects the EGFR tyrosine kinase domain to the extracellular domain through the juxtamembrane domain. The tyrosine kinase domain is responsible for autophosphoryation of tyrosine residues on the C-terminal regulatory domain of EGFR itself upon EGFR stimulation (reviewed in Burgess *et al.*, 2003). The phosphorylated tyrosine (pY) residues in the EGFR are recognized by SH2-domains and PhosphoTyrosine-Binding (PTB) domains in other proteins. This leads to recruitment of and phosphorylation of intracellular substrates. The best characterized signaling pathway activated by the EGFR is the ras-MAPK pathway. The phosphorylated residues pY1068, pY1148 and pY1173 are considered to be the major phosphorylation sites, whereas pY992, pY1045 and pY1086 are considered minor

phosphorylation sites (Downward *et al.*, 1984; Downward *et al.*, 1985; Hsuan *et al.*, 1989; Margolis *et al.*, 1989; Walton *et al.*, 1990; Levkowitz *et al.*, 1999).

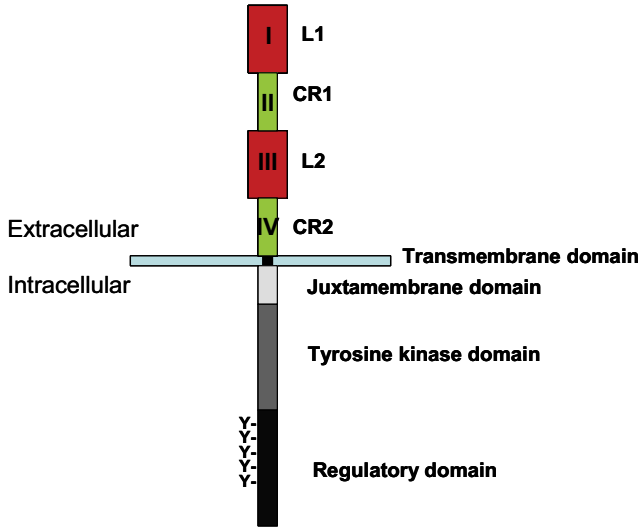

Figure 1. Structural organization of the EGFR. Extracellularly, the EGFR contains a 620 aa ligand binding domain that again is built of four domains (I-IV). The transmembrane domain spans the plasma membrane, and the juxtamembrane domain connects the EGFR tyrosine kinase domain to the extracellular domain. The regulatory domain lies C-terminally, and has multiple tyrosine residues which are phosphorylated upon activation of the EGFR.

The growth factor peptides EGF, Transforming Growth Factor alpha (TGF-α) and amphiregulin (AR) bind specifically to the EGFR (Riese and Stern, 1998). Betacellulin, Heparin-Binding EGF (HB-EGF) and epiregulin (EPR) also bind EGFR, but not exclusively. These ligands also bind ErbB4. Within the EGFR family, the different family members form homodimers, but also heterodimers, with ErbB2 as the preferred dimerization partner. The events leading to dimerization of EGFR upon ligand binding are now well understood, as the crystal structure of the extracellular domain of EGFR bound to ligand has been solved (Garrett *et al.*, 2002; Ogiso *et al.*, 2002). Upon binding ligand, the extracellular domain changes conformation from a closed to an extended configuration, thereby freeing a dimerization loop and allowing receptor dimerization (Ogiso *et al.*, 2002; Burgess *et al.*, 2003; Ferguson *et al.*, 2003) (see Figure 2). As the

family members are activated by different ligands and also stimulate different signaling pathways intracellularly, the heterodimerization allows for a complex system of EGFR

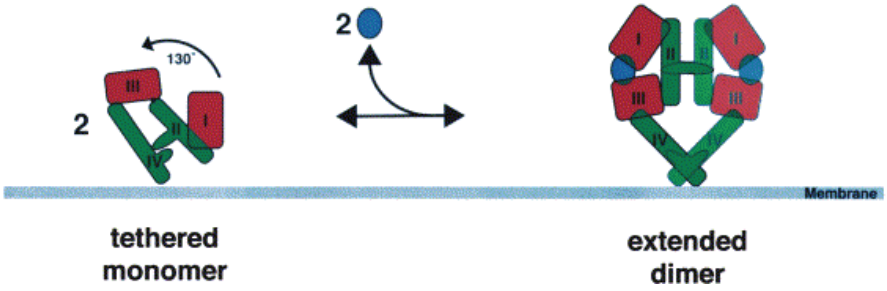

Figure 2. Conformational change in the ligand binding region of EGFR upon ligand binding and dimerization. Without bound ligand the EGFR extracellular region holds an autoinhibited configuration where the dimerization interface (domain II) is hidden by interactions with domain IV(Holbro *et al.*, 2003). EGF binding causes rotation of domain I towards domain III, freeing domain II from interactions with domain IV. Upon ligand binding, each monomer in the resulting dimer now holds an extended conformation allowing interactions between two dimerization arms in the II domains. The figure is from Bache *et al.*, 2004, and the figure legend is modified.

family receptor activation and signaling (Holbro *et al.*, 2003). As shown in Figure 3, EGFR can dimerize both with itself, and with ErbB2, leading to activation and signaling. ErbB2 is the preferred dimerization partner for all the EGFR family receptors, but this receptor has no ligand and relies on dimerization with another ligand-bound member of the EGFR family for activation. Recently, the crystal structure of the extracellular domain of ErbB2 has also been resolved, revealing a possible explanation for the preference for this receptor in dimerization. ErbB2's extracellular domain differs from that of the EGFR in having a fixed conformation resembling the ligand-activated state, thereby allowing dimerization independent of ligand binding (Cho *et al.*, 2003). ErbB3 has impaired kinase activity, and ErbB3 homodimers do not signal (Guy *et al.*, 1994)

Increased activation of the EGFR and increased expression of ErbB2 is strongly related to development of cancer. The EGFR was the first cell-surface receptor that was linked to cancer (de Larco and Todaro, 1978). Increased activation of the EGFR can arise through mutations, overexpression or stimulation of the EGFR by autocrine loops (Yarden and Sliwkowski, 2001). For controlled signaling from the EGFR, a rapid receptor inactivation

is necessary upon activation. An important pathway in this inactivation of the EGFR is by removing the EGFR from the plasma membrane by endocytosis.

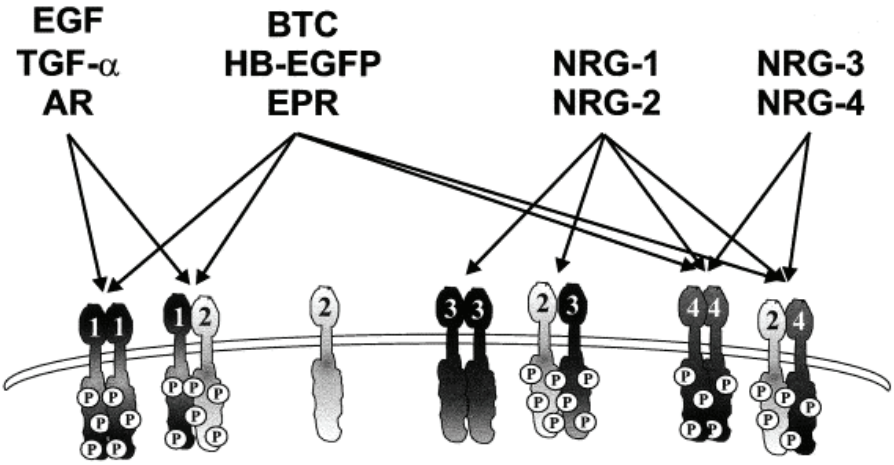

Figure 3: Ligand binding and dimerization of ErbB receptors. Upon ligand binding, EGFR family receptors dimerize into homo- or heterodimers. EGFR can dimerize with itself or with ErbB2. ErbB2 is the preferred dimerization partner for all the ErbBs, but ErbB2 has no ligand of its own and thus relies on dimerization for activation. The ErbB3 receptor has impaired kinase activity, and ErbB3 homodimers do not signal. The figure is from Traub, 2003, and the figure legend is modified.

Endocytosis

The interior of cells is confined by a biological membrane that functions to separate the interior of the cells from the environment. Small molecules such as amino acids, sugars and ions are translocated into the cell through protein channels or active pumps. Larger molecules, however, enter the cells by means of endocytosis (reviewed in Conner and Schmid, 2003), where the molecules are enclosed by the plasma membrane, pinching off to form a vesicle that transports the molecules into the cell interior (see Figure 4).

There are different forms of endocytosis. Phagocytosis is the uptake of very large particles (>50 μm) (reviewed in Aderem and Underhill, 1999) and occurs in specialized cells such as the macrophages, monocytes and neutrophils in mammals. Phagocytosis includes the uptake of bacteria, dead tissue and small particles, and is important in control of inflammation by the immune system. Pinocytosis is uptake of fluid and solutes only. A major pathway is via macropinocytosis, however, solutes and fluid can also be

internalized by clathrin-mediated endocytosis, caveolin-mediated endocytosis and clathrin- and caveolin-independent endocytosis.

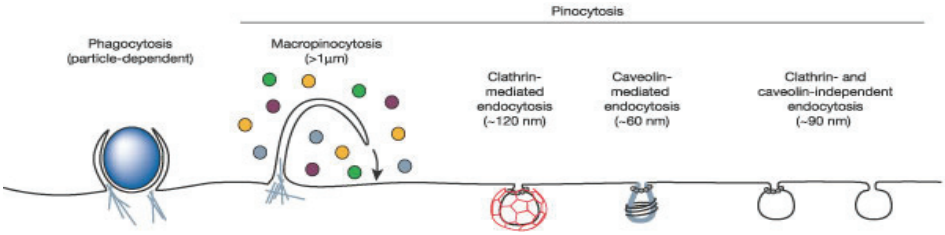

Figure 4. Different routes of endocytosis. Large particles are taken up by phagocytosis, while fluids and smaller partles are taken up by pinocytosis. This can occur either through macropinocytosis (protrusions of the plasma membrane) or by formation of vesicles by invagination of the plasma membrane. The major route of endocytosis is clathrin-mediated endocytosis, where a clathrin-cage encloses the forming vesicle. Endocytosis can also occur independently of clathrin, often by means of caveolin-mediated endocytosis, where uptake happens through vesicles enriched in caveolin. There is also evidence for other routes of internalization independent of both clathrin and caveolin. At least two different pathways have been described, one dependent on dynamin, and one independent on dynamin. The figure is from Haucke, 2005, and the figure legend is modified.

Clathrin-mediated endocytosis

The best characterized portal of entry for nutrients and receptor ligands into cells is clathrin-mediated endocytosis (reviewed in Kirchhausen, 2000; reviewed in Conner and Schmid, 2003) . The main components of clathrin-coated pits are clathrin triskelia and their adaptors. From these coated pits the membrane buds inwards and pinches off from the plasma membrane thereby forming clathrin-coated vesicles. Upon formation of the coated vesicle, clathrin is removed and the uncoated vesicle moves inwards and eventually fuses with other uncoated vesicles and/or preexisting early endosomes. While some receptors like the Low-Density Lipoprotein Receptor (LDLR) and the Transferrin Receptor (TfR) are constantly recruited to clathrin-coated pits, others become incorporated only after activation of the receptor (reviewed in Mukherjee *et al.*, 1997). Receptors are recruited to clathrin-coated pits by interactions with adaptors, like the Adaptor Protein complex 2 (AP-2) in the case of the TfR (Mukherjee *et al.*, 1997).

Clathrin

Coated vesicles were first observed in 1964 (Roth and Porter, 1964) by means of electron microscopy. The structure was further described as a lattice consisting of pentagons and hexagons (Kanaseki and Kadota, 1969; Kadota and Kadota, 1973; Crowther *et al.*, 1976),

and the major component of the coat was later demonstrated to be clathrin(Pearse, 1975, 1976). Clathrin assembles into a structure called a triskelion, a three-legged structure built by three heavy and three light chains of clathrin (Ungewickell and Branton, 1981). The Clathrin Heavy Chain (CHC) polypeptide contains different functional regions (see Figure 5). The N-terminal domain is a globular ß-propeller. The globular domain is important in binding other proteins, while the distal and proximal segments are important for self-assembly of the clathrin cage. The proximal segment also mediates binding of Clathrin Light Chain (CLC) (reviewed in Mousavi *et al.*, 2004). The CLC exists in two isoforms, LCa and LCb. These appear to be randomly recruited to clathrin triskelia and are believed to be involved in CHC trimerization (Chu *et al.*, 1996; Huang *et al.*, 1997). Additionally CLC is believed to negatively contribute to the regulation of self-assembly of clathrin (Ybe *et al.*, 1998) and has also been proposed to be involved in recruitment of the actin polymerization machinery (Newpher *et al.*, 2006).

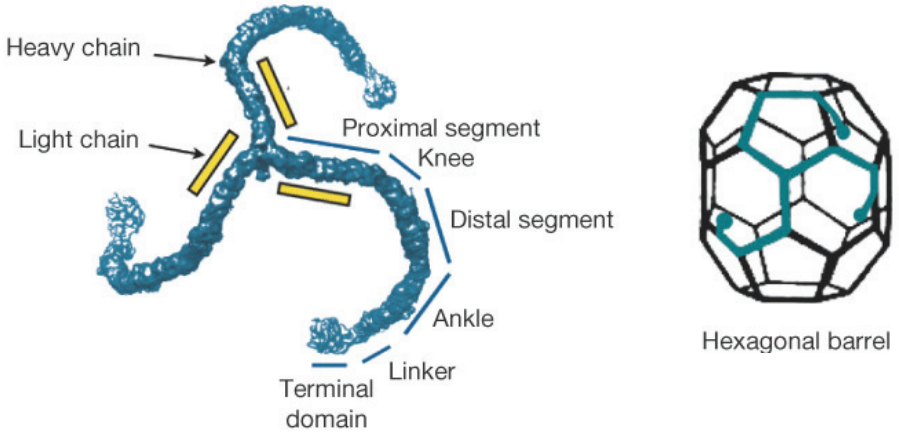

Figure 5. Structure of the clathrin triskelion and the hexagonal clathrin-barrel. **A** Model of the clathrin triskelion composed of three heavy chains (blue) and three light chains (yellow). The heavy chain contains five distinct regions: The globular N-terminal domain, the more flexible linker segment, the "ankle", the distal and the proximal segment separated by the "knee"**. B** The smallest symmetrical form of clathrin triskelia is the hexagonal barrel, here shown with a single triskelion highlighted in blue. The figure is adapted from Fotin *et al.*, 2004, and the figure legend is modified.

AP-2

In addition to clathrin, the clathrin-coated pit also contains adaptor proteins. Adaptor proteins are responsible for the selection of cargo proteins in clathrin-mediated endocytosis (reviewed in Traub, 2003) by their ability to bind both cargo molecules and clathrin. The major adaptor protein complex in clathrin-mediated endocytosis is AP-2, and AP-2 is believed to be the key protein complex responsible for coated pit formation. It is still unclear, however, whether AP-2 is actually critical for all clathrin-mediated endocytosis (Hinrichsen *et al.*, 2003; Motley *et al.*, 2003; Rappoport *et al.*, 2006). The AP-2 complex is composed of four subunits, α, ß2, µ2 and δ2. The two large subunits, α and ß2 are each composed of a N-terminal domain called the head or trunk domain, and a globular C-terminal domain called the appendage, or the ear, domain. These two distinct domains are connected through the flexible hinge domain. AP-2 is targeted to the plasma membrane through its α-subunit which interacts with phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) or phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) in the membrane (Gaidarov *et al.*, 1996; Gaidarov and Keen, 1999). Additionally, the ear domain of the α-subunit has been shown to interact with regulators of vesicle formation. Such regulators are epsin, Eps15 (EGFR-pathway substrate-15) and amphiphysin (Slepnev and De Camilli, 2000). A clathrin box in the hinge domain and an additional domain near the ear domain of the ß2-subunit facilitates direct binding to clathrin (Ahle and Ungewickell, 1989; Kirchhausen *et al.*, 1989; Mousavi *et al.*, 2004) and facilitates clathrin assembly. The ß2-subunit may also be involved in cargo recognition (Rapoport *et al.*, 1998). Cargo is recruited through interactions between sorting motifs in protein cargo and the AP-2 complex. The most important subunit for cargo selection is the µ2-subunit, which interacts with tyrosine-based sorting signals (YXXΦ, where Φ is a bulky hydrophobic residue) and dileucine sorting signals within the cytosolic domain of integral membrane protein receptors (Aguilar *et al.*, 1997). In addition, the µ2-subunit contains a phosphoinositide-binding site (Rohde *et al.*, 2002). The current view of AP-2 function in clathrin-mediated endocytosis is that AP-2 initially is recruited to an assembly site at the plasma membrane through its cargo- and membrane-binding abilities. It is then self-associated to form clusters which in turn recruit and assemble clathrin (reviewed in Mousavi *et al.*, 2004). In addition to AP-2, the tetrameric adaptor protein complexes AP-

1, AP-3 and AP-4 are also involved in clathrin-coat formation. Whereas AP-2 is primarily involved in clathrin assembly at the plasma membrane, AP-1, AP-3 and AP-4 mediate sorting events at the *trans*-Golgi network (TGN) or endosomes (reviewed in Boehm and Bonifacino, 2001).

Additional adaptor proteins in clathrin-mediated endocytosis

In addition to AP-2, other adaptor proteins also appear to be important in clathrin-mediated endocytosis (reviewed in Mousavi *et al.*, 2004). AP180 localizes to synapses (Perry *et al.*, 1992), while CALM (Clathrin Assembly Lymphoid Myeloid leukemia protein) is ubiquitously expressed (Dreyling *et al.*, 1996). Each protein has a $PI(4,5)P_2$ binding domain and both bind to AP-2 and clathrin. Both AP-2 and CALM/AP180 are able to stimulate clathrin assembly by them selves, but the interaction between AP-2 and CALM/AP180 increases the ability of AP-2 to assemble clathrin (Hao *et al.*, 1999). ß-arrestin is another sorting adaptor involved in clathrin-mediated endocytosis. ß-arrestin binds to $PI(4,5)P_2$ (Gaidarov *et al.*, 1999a), clathrin and AP-2 (Goodman *et al.*, 1996; Laporte *et al.*, 1999) and is involved in endocytosis of G Protein-Coupled Receptors (GPCRs) (reviewed in Marchese *et al.*, 2003a). Upon activation and phosphorylation of GPCRs and engagement of ß-arrestin, ß-arrestin is recruited to preexisting sites of clathrin assembly where it promotes rapid endocytosis of GPCRs (reviewed in Traub, 2003). Dab2 (Disabled-2) is another protein suggested to act as an adaptor in clathrin-mediated endocytosis (Traub, 2003). Dab2 binds $PI(4,5)P_2$ and clathrin (Mishra *et al.*, 2002). Dab2 interacts with AP-2 and can also interact with non-tyrosine-phosphorylated motifs in the cytoplasmic tail of LDLR (Oleinikov *et al.*, 2000; Morris and Cooper, 2001).

Epsin is localized to clathrin-coated pits (Stang *et al.*, 2004; Hawryluk *et al.*, 2006) and has been found to be involved in clathrin-mediated endocytosis (Chen *et al.*, 1998; Wendland *et al.*, 1999). Whereas some authors have proposed that epsin functions as an adaptor in clathrin-mediated endocytosis (reviewed in Wendland, 2002), others have shown that epsin is involved in the formation of membrane curvature within clathrin-coated pits. Epsin contains an ENTH (epsin amino-terminal homology) domain that binds to $PI(4,5)P_2$ (Itoh *et al.*, 2001; Ford *et al.*, 2002) and through this interaction is proposed

to facilitate membrane curvature by insertion of an epsin α-helix into the inner leaflet of the membrane lipid bilayer (Ford *et al.*, 2002). Epsin binds both clathrin and AP-2 (Chen *et al.*, 1998; Hussain *et al.*, 1999; Owen *et al.*, 1999; Rosenthal *et al.*, 1999; Traub *et al.*, 1999; Drake *et al.*, 2000), and epsin has multiple Ubiquitin Interacting Motifs (UIMs) that recently were shown that to preferentially bind polyubiquitin chains (Hawryluk *et al.*, 2006). Given these capabilities, epsin has been proposed to function as an adaptor for sorting of ubiquitinated cargo for clathrin-mediated endocytosis (Barriere *et al.*, 2006; Duncan *et al.*, 2006; Hawryluk *et al.*, 2006; Sorkina *et al.*, 2006). Epsin was first described as an Eps15 interacting protein (McPherson *et al.*, 1998). Eps15 is an AP-2 binding (Benmerah *et al.*, 1996) protein with conserved N-terminal EH (Eps15 homology) domains through which Eps15 binds epsin (Chen *et al.*, 1998). Eps15 also binds polyubiquitin and is suggested to act in partnership with epsin to sort polyubiquitinated cargo into clathrin-coated vesicles (Hawryluk *et al.*, 2006).

Sorting signals

The sorting signal normally rests in the cytoplasmic part of the receptor to be identified and the most studied sorting signals are the tyrosine-based (consensus motif NPXY or YXXΦ) and the dileucine based (consensus motifs [DE]XXXL[LI] or DXXLL) sorting signals (reviewed in Bonifacino and Traub, 2003). In the case of the TfR the tetrapeptide is YXRF (Jing *et al.*, 1990; McGraw and Maxfield, 1990), a tyrosine-based internalization signal of the YXXΦ type that binds directly to the μ2-subunit of AP-2. Binding of μ2 to tyrosine-based sorting signals is proposed to be dependent upon phosphorylation of μ2, likely mediated by the kinases AAK1 (Adaptor-Associated Kinase 1) and GAK (cyclin-G-Associated protein Kinase) (Umeda *et al.*, 2000; Olusanya *et al.*, 2001; Collins *et al.*, 2002; Conner and Schmid, 2002; Korolchuk and Banting, 2002; Ricotta *et al.*, 2002; Conner *et al.*, 2003; Sorkin, 2004). Other receptors are believed to make use of additional connector proteins coupling their sorting signals to the clathrin coat. The LDLR does not have the YXXΦ sorting signal of TfR. LDLR contains a distinct signal, FxNPxY, and the LDLR does not appear to depend on AP-2 for internalization to the same degree as does the TfR (Traub, 2003). ARH (Autosomal Recessive Hypercholesterolemia) and Dab2 have been suggested to interact with the FxNPxY of LDLR, in addition to AP-2 and clathrin, thereby recruiting LDLR to coated

pits (He *et al.*, 2002; Mishra *et al.*, 2002; Nagai *et al.*, 2003; Sorkin, 2004). Whether or not the internalization of EGFR is dependent on AP-2 is under discussion. EGFR interacts with AP-2 (Sorkin and Carpenter, 1993; Sorkin *et al.*, 1995), but the internalization rate is not significantly affected by mutations inhibiting its interaction with AP-2 (Nesterov *et al.*, 1995). Furthermore, some have reported that downregulation of the μ2 subunit of AP-2 did not affect EGFR internalization (Motley *et al.*, 2003), whereas others have found that μ2 is important for EGFR downregulation (Huang *et al.*, 2004). There has also been contradiction regarding the importance of the α2 subunit of AP-2 in internalization of the EGFR (Hinrichsen *et al.*, 2003; Motley *et al.*, 2003; Huang *et al.*, 2004; Johannessen *et al.*, 2006). Receptors are believed to be recruited into preexisting clathrin-coated pits. However, it has been shown that the EGFR is able to induce the formation of new clathrin-coated pits (Johannessen *et al.*, 2006).

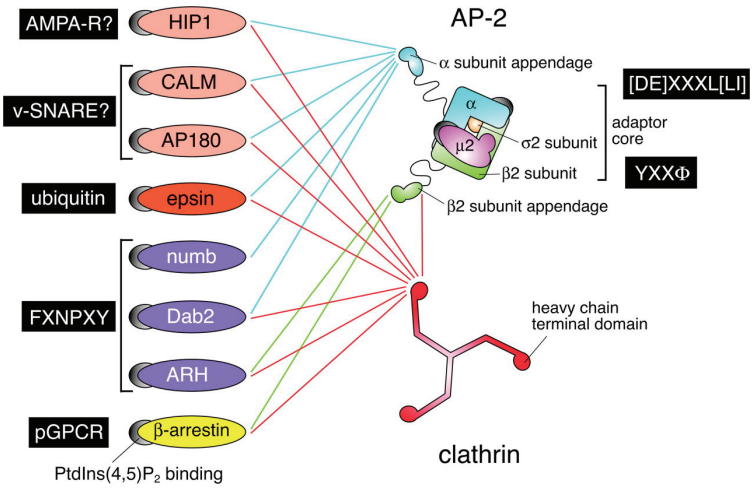

Figure 6. Interactions between the different subunits of the AP-2 complex, clathrin and other possible adaptors/connector proteins. AP-2 interacts directly with proteins with sorting signals YXXΦ and (DE)XXXL(LI). Other signals requires the help of connector proteins for recruitment to clathrin-coated pits. Epsin is believed to recruit ubiquitinated proteins to clathrin-coated pits through interactions with both clathrin and the appendage domain of the α-subunit of AP-2. Dab2 and ARH are believed to help recruit proteins with the sorting signal FXNPXY to clathrin-coated pits through interactions with clathrin and AP-2. Dab2 interacts with the appendage domain of the α-subunit of AP-2, while ARH interacts with the appendage domain of the ß-subunit. ß-arrestin connects GPCRs to clathrin-coated pits through interactions with both clathrin and AP-2. The figure is from Traub, 2003, and the figure legend is modified.

Ubiquitination as internalization signal for EGFR

Requirement of AP-2 in internalization of the EGFR is questioned and the mechanisms for recruitment of EGFR to clathrin-coated pits are elusive. Lately, the focus for EGFR recruitment to clathrin-coated pits has been on ubiquitination. Ubiquitin is a 76 aa peptide, which upon a chain reaction involving E1, E2 and E3 enzymes becomes covalently attached to lysine residues on target proteins. The ubiquitin-activation enzyme (E1) binds free ubiquitin. In the case of HECT (Homologous to E6AP Carboxy Terminus)-domain ligases, ubiquitin is then transferred via an E2 conjugating enzyme, to an E3 ligase enzyme. The E3 also binds the target protein, and catalyzes the covalent attachment of ubiquitin to the target. RING (Really Interesting New Gene)-domain E3 ubiquitin ligases, however, are not believed to bind ubiquitin but to mediate direct transfer from the E2 conjugating enzyme to the target protein (reviewed in Weissman, 2001). The binding of EGF results in dimerization of the EGFR and subsequent activation of its kinase domain and autophosphorylation of tyrosine residues in the cytoplasmic tail. SH2-domain containing proteins recognize and bind phosphorylated tyrosine residues, and the autophosphorylation of EGFR in dimers is followed by recruitment of different proteins containing Src Homology 2 (SH2)-domains (reviewed in Burgess *et al.*, 2003). An SH2-domain containing protein of special interest in EGFR endocytosis is the ubiquitin ligase Cbl. Cbl binds both directly and indirectly to activated EGFR (Galisteo *et al.*, 1995; Lupher *et al.*, 1996; Waterman *et al.*, 2002) and acts as a negative regulator of EGFR signaling (reviewed in Thien and Langdon, 2001). The EGFR has been found to be ubiquitinated upon EGF stimulation (Galcheva-Gargova *et al.*, 1995). Cbl has been demonstrated to be responsible for this ubiquitination (Levkowitz *et al.*, 1998; Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999; Waterman *et al.*, 1999; Yokouchi *et al.*, 1999; Lill *et al.*, 2000), and it has been suggested that ubiquitination of the EGFR acts as a signal for endocytosis of the EGFR (Levkowitz *et al.*, 1998; Miyake *et al.*, 1998). Cbl is an E3 ubiquitin ligase of the RING-finger family, and there are three Cbl proteins in mammals: c-Cbl, Cbl-b and Cbl-3 (also called Cbl-c). c-Cbl, Cbl-b and Cbl-3 have Tyrosine Kinase Binding (TKB) domains, through which Cbl proteins can interact directly with the EGFR at pY1045 (Levkowitz *et al.*, 1999). Additionally, c-Cbl and Cbl-b bind the EGFR adaptor protein Grb2 (Growth factor

receptor-bound protein 2) via a proline-rich region in c-Cbl/Cbl-b and one of the SH3 domains of Grb2 and is believed to be recruited to the EGFR indirectly through such interactions (see Figure 7). Both direct and indirect interaction of Cbl proteins with EGFR mediate ubiquitination of the EGFR (Levkowitz *et al.*, 1996; Waterman *et al.*, 2002).

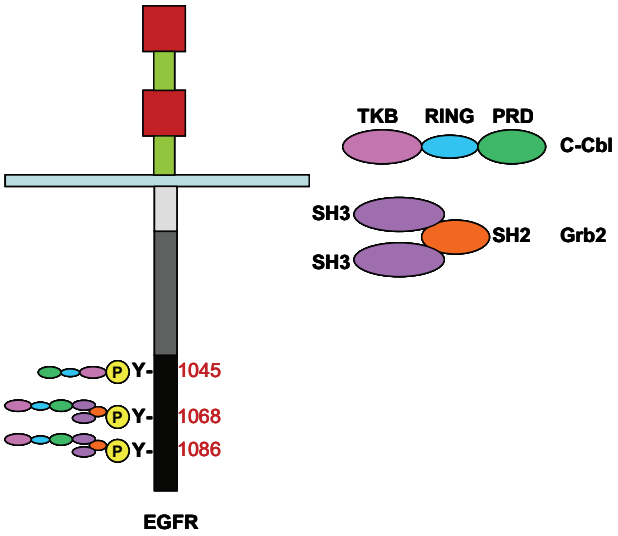

Figure 7. c-Cbl is recruited to EGFR directly through pY1045 and indirectly via Grb2 to EGFR through pY1068 or pY1086. c-Cbl contains a binding site for phosphorylated EGFR tyrosine residue 1045 within the tyrosine kinase binding (TKB) domain. c-Cbl is also able to interact with an SH3-domain of Grb2 through its proline rich domain (PRD). Grb2 interacts with EGFR through phosphorylated tyrosine residues 1068 and 1086, and recruits c-Cbl to EGFR also through this interaction.

In addition to different protein-protein interaction domains, Cbl proteins contain a cysteine rich RING finger domain which has been shown to be responsible for the ubiquitin ligase activity. The domain is required for recruitment of E2 enzymes, and functions together with the linker sequence that connects the TKB domain and the RING finger domain in this recruitment (Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999; Yokouchi *et al.*, 1999; Zheng *et al.*, 2000). The E3 activity is regulated by phosphorylation of residues Y368 and Y371 in c-Cbl, and phosphorylation probably results in conformational changes in c-Cbl favoring E3 activity (Levkowitz *et al.*, 1999; Kassenbrock and Anderson, 2004). Additionally, c-Cbl activity is proposed to be

regulated by ubiquitination. c-Cbl mediates ubiquitination both of c-Src and of c-Cbl itself (Yokouchi *et al.*, 2001).

Downregulation of the EGFR is regulated at several steps in endocytosis, both by sorting into clathrin-coated pits at the plasma membrane and by sorting for degradation or recycling at the early endosomes. Whether or not c-Cbl-mediated ubiquitination of the EGFR is important at the internalization step in endocytosis has been discussed. Inhibition of EGFR ubiquitination by overexpression of the Cbl-binding protein Sprouty, known to inhibit Cbl's interactions with E2 enzymes (Fong *et al.*, 2003), was found to block the progression of the EGFR into clathrin-coated pits and to block endocytosis of the EGFR (Stang *et al.*, 2004). However, others have found that Cbl-mediated ubiquitination is required for EGFR degradation, but not for internalization (Duan *et al.*, 2003). Also, it has been shown that overexpression of c-Cbl does not affect EGFR internalization, but increases EGFR degradation only (Levkowitz *et al.*, 1998). In the same study, overexpression of the oncogenic v-Cbl, a Cbl mutant able to bind pY1045 in the EGFR, but lacking the RING finger and the proline-rich domain, did not affect internalization of the EGFR. This Cbl mutant, however, increased receptor recycling. In addition, EGFR has been shown to be internalized independently of activation, by use of the specific EGFR tyrosine kinase inhibitor AG-1478 together with EGF (Wang *et al.*, 2002). An EGFR mutant unable to bind Cbl directly (Y1045F) was not inhibited in internalization (Jiang and Sorkin, 2003). Finally, in a CHO cell line with a temperature-sensitive E1 ubiquitin-activating enzyme, EGFR downregulation, but not internalization, was impaired. In this last study it was also proposed using $Cbl^{-/-}$ mouse embryonic fibroblast cell lines that endogenous Cbl is essential for ligand-induced ubiquitination and efficient degradation of EGFR, but not for internalization (Duan *et al.*, 2003).

Monoubiquitination vs polyubiquitination

As already described, ubiquitin is covalently attached to lysine residues on target proteins. However, also ubiquitin carries lysine residues, and these lysines serve as sites for self-conjugation. This leads to chains of multiply linked ubiquitin peptides, usually linked through lysine residue 48 (Lys48) but also Lys63, Lys11 and Lys29-chains are

known to exist (reviewed in Weissman, 2001). The Lys48-linked chains tags proteins for proteosomal degradation and are important signals for the turnover of many proteins in eukaryotic cells. Lys63-chains are known to be important in DNA repair, but have also been shown to be involved in targeting proteins for endocytosis and for vacuolar degradation of transporters in yeast. The ubiquitination of EGFR results in a "smear" of the EGFR band when doing Western blot, representing EGFR of higher molecular weight. This can represent both polyubiquitination as well as multiubiquitination patterns. The activation of EGFR was initially believed to result in polyubiquitination of the receptor (Galcheva-Gargova *et al.*, 1995). In yeast, however, monoubiquitination was initially proposed to be the signal for endocytosis of plasma membrane receptors (Hicke and Riezman, 1996). Monoubiquitin has been shown to be sufficient for internalization of membrane receptors in yeast (Shih *et al.*, 2000). In human cells, addition of a single ubiquitin molecule to the TfR, which is normally recycled from early endosomes, was sufficient for sorting the TfR into Hepatocyte growth factor regulated tyrosine kinase substrate (Hrs)-positive sorting microdomains on early endosomes (Raiborg *et al.*, 2002), and monoubiquitination of EGFR has been shown to be sufficient for EGFR internalization (Haglund *et al.*, 2003; Mosesson *et al.*, 2003). It is not known though, whether additional ubiquitin residues are added to these initial monoubiquitin molecules, giving polyubiquitination intracellularly. Recently, using antibodies specific for polyubiquitin and also by using mutants of ubiquitin and EGFR, multiple monoubiquitination was proposed to be sufficient for internalization and degradation of receptor tyrosine kinases (Haglund *et al.*, 2003; Mosesson *et al.*, 2003). The question whether mono- or polyubiquitination drives endocytosis is still, however, discussed. In fact, using tandem mass spectrometry, it was recently demonstrated that more than 50% of the ubiquitin on activated EGFR is in the form of polyubiquitin, mostly Lys63-linked, but also some ubiquitin linked through Lys48, Lys11 and Lys29 was observed (Huang *et al.*, 2006). Is has also been shown that the UIM of epsin, an adaptor protein proposed to be important for internalization of ubiquitinated cargo, preferentially binds to polyubiquitin (Hawryluk *et al.*, 2006).

Dynamin

Dynamin is a large GTPase important for both clathrin-dependent and clathrin-independent endocytosis, but its exact function in endocytosis is still partly unclear. Dynamin contains four conserved domains (see Figure 8).

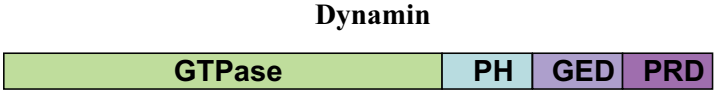

Figure 8. Structural organization of dynamin. Dynamin contains a GTPase domain through which it binds GTP or GDP, a PH domain through which dynamin interacts with $PI(4,5)P_2$ of membranes, a GTPase effector domain (GED) through which dynamin self-assembles and activates its GTPase activity and a proline-rich domain (PRD) through which dynamin interacts with a vast number of SH3-domain-containing proteins.

At the N-terminal end is a GTPase domain through which dynamin binds GTP and GDP. The pleckstrin homology (PH) domain mediates binding of dynamin to phosphoinositides, facilitating membrane binding of the protein (Salim *et al.*, 1996; Klein *et al.*, 1998). Dynamin is capable of hydrolysing GTP without the help of an extra GAP (GTPase activating protein). Indeed, dynamin contains its own GAP, the GTPase Effector Domain (GED). Self-assembly of dynamin into oligomers activates the GTPase-stimulating effect of this domain (Muhlberg *et al.*, 1997; Sever *et al.*, 1999). Through its Proline Rich Domain (PRD), dynamin is further able to interact with a number of SH3 domain containing proteins involved in endocytosis, such as amphiphysin, cortactin, endophilin, Grb2, intersectin and Src (Gout *et al.*, 1993; Miki *et al.*, 1994; Seedorf *et al.*, 1994; Grabs *et al.*, 1997; Ringstad *et al.*, 1997; Foster-Barber and Bishop, 1998; Yamabhai *et al.*, 1998; McNiven *et al.*, 2000). There is increasing evidence that dynamin is involved in the late stages of clathrin-coated vesicle formation, most likely in pinching off of clathrin-coated pits (Kosaka and Ikeda, 1983; Carter *et al.*, 1993; van der Bliek *et al.*, 1993; Damke *et al.*, 1994; Damke *et al.*, 1995; Sever *et al.*, 2000; Damke *et al.*, 2001; Narayanan *et al.*, 2005). Based on the work with the dynamin specific GTP hydrolysis inhibitor dynasore, it was recently proposed that dynamin may in fact act at two different stages in clathrin-vesicle formation, (Macia *et al.*, 2006). Using dynasore, the authors observed the arrest of clathrin-coated vesicle formation at two different stages, both

directly prior to pinching off and at an earlier stage. Treating cells with dynasore resulted in accumulation of what appeared as U-shaped pits which were partially coated, indicating halt at an early stage in clathrin-coated pit formation.

Dynamin has been demonstrated to be involved in recycling from early endosomes to the TGN (Nicoziani *et al.*, 2000) and dynamin has also been shown to be recruited to early endosomal clathrin coats upon overexpression of the endosomal sorting-protein Hrs (Raiborg *et al.*, 2001a). Recycling of the TfR has been proposed to follow two distinct pathways, one pathway where dynamin is involved in transport from the early endosome to the recycling endosome, and another pathway different from that via the recycling endosome (van Dam *et al.*, 2002). Although the importance of dynamin in receptor recycling from early endosomes has been reported, dynamin is proposed to function in endocytosis mainly on the plasma membrane. In addition to clathrin-mediated endocytosis, dynamin is important for caveolin-mediated endocytosis and has been reported to be necessary for other forms of caveolin- and clathrin-independent endocytosis. Dynamin-independent fluid phase uptake has also been reported, implying that there exist forms of endocytosis that do not rely on dynamin (reviewed in Conner and Schmid, 2003).

The actin cytoskeleton

Although the importance of the actin cytoskeleton in clathrin-mediated endocytosis is at present unclear (Fujimoto *et al.*, 2000; Engqvist-Goldstein and Drubin, 2003), there is growing evidence for the involvement of the actin cytoskeleton in clathrin-mediated endocytosis in mammalian cells (reviewed in Merrifield, 2004). In yeast, actin and actin dynamics have been shown to be important for endocytosis (Engqvist-Goldstein and Drubin, 2003). In mammalian cells the actin motor protein Myosin VI has been shown to localize to clathrin-coated vesicles and to be important for endocytosis of the TfR (Buss *et al.*, 2001). The involvement of Myosin VI in clathrin-mediated endocytosis has been proposed to occur at the stage of transport inwards in the cell (Aschenbrenner *et al.*, 2003). Actin and the regulators of actin polymerization are believed to be recruited to clathrin-coated vesicles by dynamin, as dynamin binds multiple actin-interacting proteins (reviewed in Orth and McNiven, 2003). Dynamin is proposed to recruit activators of the

Arp2/3 complex such as cortactin and Neural Wiskott-Aldrich Syndrome protein (N-WASP) to the neck of clathrin-coated pits, thereby causing actin polymerization, which can facilitate the last steps of internalization (Qualmann *et al.*, 2000; Kessels and Qualmann, 2002; Cao *et al.*, 2003; Merrifield, 2004; Merrifield *et al.*, 2004). The actin cytoskeleton is also believed to control lateral movement of clathrin-coated pits (Gaidarov *et al.*, 1999b; Santini *et al.*, 2002; Mousavi *et al.*, 2004). Lately, it has also been proposed that the actin cytoskeleton is in fact important at multiple stages of clathrin–mediated endocytosis such as coated pit formation, constriction, scission and lateral motility (Yarar *et al.*, 2005).

Phosphoinositides in endocytosis

The lipid composition of membranes is considered to be important for endocytosis. In particular, the phosphoinositides have been shown to direct membrane trafficking by recruitment of adaptors and by creating membrane specificity (reviewed in Di Paolo and De Camilli, 2006). Phosphatidylinositol (PI) is a phospholipid found at the cytosolic face of membranes, and can be phosphorylated in three different positions of its inositol ring, giving rise to seven different phosphoinositides, each with a unique subcellular localization (see Figure 9). $PI(4,5)P_2$ has been shown to be enriched at the plasma membrane, and $PI(4,5)P_2$ mediates recruitment of AP-2 to the plasma membrane in clathrin-mediated endocytosis (Gaidarov *et al.*, 1999b; Santini *et al.*, 2002; Mousavi *et al.*, 2004). In fact, all the known adaptors in clathrin-mediated endocytosis can bind $PI(4,5)P_2$. Additionally, phosphoinositides also recruit both guanine nucleotide exchange factors (GEFs) and GAPs. The Rab and Arf classes of small GTPases are important for defining intracellular membranes (reviewed in Behnia and Munro, 2005), and their activity is regulated by GAPs and GEFs. GTPases can in turn control phosphoinositide-metabolizing enzymes. Phosphoinositides are also involved in recruitment of elements of the cytoskeleton to membranes. $PI(4,5)P_2$ and the small GTPase Cdc42 bind N-WASP. Together, the binding of $PI(4,5)P_2$ and Cdc42 to N-WASP triggers binding of N-WASP to the Arp2/3 complex and activation of the Arp2/3 complex (Rohatgi *et al.*, 2000). This, in turn, activates actin polymerization. In addition to this, $PI(4,5)P_2$ has been shown to bind dynamin (Achiriloaie *et al.*, 1999; Lee *et al.*, 1999; Vallis *et al.*, 1999). Another phosphoinositide, PI(3)P, is found mainly on early endosomes and contributes to

lysosomal sorting by recruitment of PI(3)P-binding proteins. PI(3)P can be formed by dephosphorylation of PI(3,4,5)P_3, but most often by phosphorylation of PI by PI(3)kinase. Less is known about phosphoinositide PI(3,5)P_2, but it has been coupled to protein trafficking in the later steps of endocytosis (reviewed in Michell *et al.*, 2006).

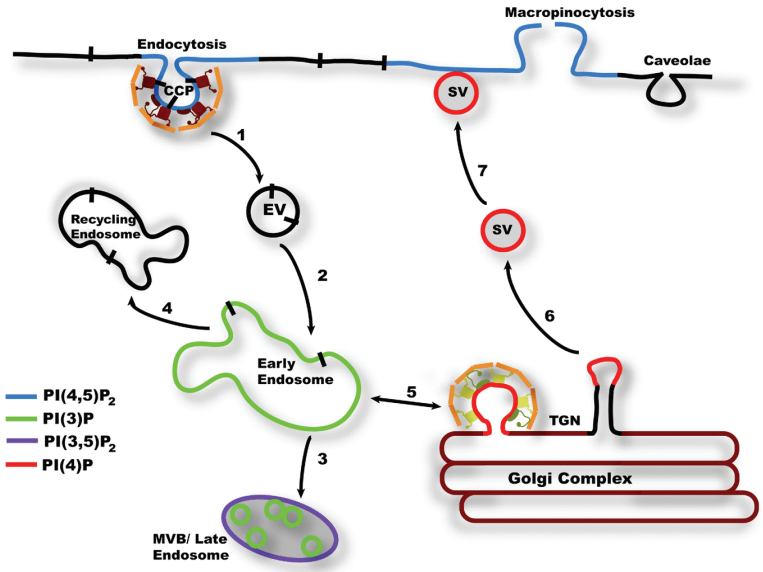

Figure 9. Intracellular distribution of the different phosphoinositides in cell membranes. PI(4,5)P_2 is mainly found in the plasma membrane, whereas PI(3)P is predominantly located to early endosomal membranes. At later sorting endosomes or MVBs both PI(3)P and the less studied PI(3,5)P is found. PI(4)P is mainly enriched in the trans-Golgi network (TGN). The figure is from Burgess *et al.*, 2003.

Endosomal sorting of ubiquitinated cargo

After budding of clathrin-coated pits, the coat disassembles, and the uncoated vesicle with its cargo fuses with the early endosome. Here internalized receptors are sorted into different microdomains for recycling back to the plasma membrane, for transport to the TGN, or to the lysosome for degradation. Cbl-mediated ubiquitination of the EGFR has been shown to be important for the sorting of EGFR at early endosomes (Levkowitz *et al.*, 1999; Longva *et al.*, 2002; Duan *et al.*, 2003). The process of sorting to the lysosome for degradation involves recognition of ubiquitinated cargo by an endosomal sorting

machinery and subsequent internalization of retained cargo into intracellular vesicles of multivesicular bodies (MVBs) and further transport to the lysosome (reviewed in Raiborg *et al.*, 2003). Recently, much information has emerged on the machinery responsible for this sorting, and the focus has been on Hrs and the Endosomal Sorting Complex Required for Transport (ESCRT) complexes. Hrs has been shown to localize to the membrane of early endosomes, and it is homologous to the yeast protein Vps27p known to be important in protein traffic through a pre-vacuolar compartment (Komada *et al.*, 1997). Later, Hrs was shown to localize to microdomains on the limiting membrane of early endosomes, identified by the presence of flat, bilayered coats. Often, inwards membrane budding is observed at the edge of these coats (Raiborg *et al.*, 2001a; Raiborg *et al.*, 2002; Sachse *et al.*, 2002). These coats were also shown to be enriched in EGFR, but not in TfR (Raiborg *et al.*, 2002; Sachse *et al.*, 2002). Hrs is recruited to early endosomes by the specific interactions between its FYVE domain (a zinc finger domain, named after Fab1, YOTB/ZK632.12, Vac1 and EEA1) and PI(3)P in the limiting membrane of the endosome (Urbe *et al.*, 2000; Raiborg *et al.*, 2001b). Another domain in Hrs, a coiled-coil domain, is also believed to be involved in membrane microdomain-binding specificity (Raiborg *et al.*, 2001b). Recently, it was demonstrated that clathrin is important in recruiting Hrs to the specialized microdomains (Raiborg *et al.*, 2006). Additionally, Hrs contains a VHS (Vps27, Hrs and STAM) domain. Such domains have been shown to interact directly with receptors (Nielsen *et al.*, 2001). The protein also contains a UIM which is capable of binding monoubiquitin *in vitro*, but actually prefers binding to polyubiquitin (Bishop *et al.*, 2002; Polo *et al.*, 2002; Raiborg *et al.*, 2002). Recently, the structure of the UIM in Hrs bound to ubiquitin was solved, and it was demonstrated that the UIM of Hrs in fact binds two ubiquitin molecules, on two sides of an α-helix. Both binding sites were shown to be necessary for efficient protein sorting to the degradative pathway (Hirano *et al.*, 2006). Overexpression of Hrs has been shown to inhibit degradation of the EGFR (Chin *et al.*, 2001; Raiborg *et al.*, 2001a; Bishop *et al.*, 2002) and this could potentially be an effect of clathrin clustering and not directly of Hrs itself. Hrs has been shown to bind Eps15 and STAM (Signal-Transducing Adaptor Molecule), and it has been shown that these proteins form a complex. It has also been shown that Hrs recruits STAM to the endosomal membrane, and that Hrs, STAM and Eps15 co-localize

with ubiquitinated proteins in clathrin-positive microdomains on early endosomes (Asao *et al.*, 1997; Bache *et al.*, 2003). As both Hrs, STAM and Eps15 bind ubiquitinated proteins, they have been suggested to function together in the capture of ubiquitinated proteins for sorting into MVBs (Bache *et al.*, 2003). Also, as Hrs has been shown to bind clathrin and recruit clathrin to early endosomes (Raiborg *et al.*, 2001a), it has been suggested that clathrin coats recruited by Hrs concentrate receptors in microdomains prior to the invagination of the membrane in formation of inner MVB vesicles (Raiborg and Stenmark, 2002). Hrs is ubiquitinated itself, and must be deubiquitinated for receptor sorting to the degradative pathway. This ubiquitination status is proposed to be regulated by Vps4 (Marchese *et al.*, 2003b).

After binding of ubiquitinated cargo by the Hrs/STAM (Vps27/Hse1p in yeast) complex, Hrs is believed to recruit the ESCRT-I complex through the ESCRT-I subunit Tsg101 (Vps23 in yeast). Cargo is then transferred to Tsg101 in the ESCRT-I complex. (reviewed in Slagsvold *et al.*, 2006). ESCRT-I is composed of three subunits, namely Vps23, Vps28 and Vps37. Downstream of ESCRT-I, ESCRT-II takes over the ubiquitinated cargo (Raiborg *et al.*, 2003). ESCRT-II consists of the three subunits Vps22, Vps25 and Vps36, and ESCRT-II is able to bind ubiquitin through Vps36 (Raiborg *et al.*, 2003). After transfer of ubiquitinated cargo to ESCRT-II, the ESCRT-III complex is recruited to early endosomes. This complex has two subcomplexes, Snf-Vps20 and Vps2-Vps24 and has been proposed to function in the final steps of inner-vesicle scission on early endosomes (Raiborg *et al.*, 2003).

The limiting membrane of early endosomes contains multiple coated domains (Murk *et al.*, 2003), and it has been shown that endosomal coats are both Hrs-positive and -negative, as well as clathrin-positive and -negative (Prekeris *et al.*, 1999; Sachse *et al.*, 2004). It is possible that the Hrs-negative coats are in fact formed by the ESCRT complexes, and are involved in the final steps of endosomal sorting for the degradative pathway.

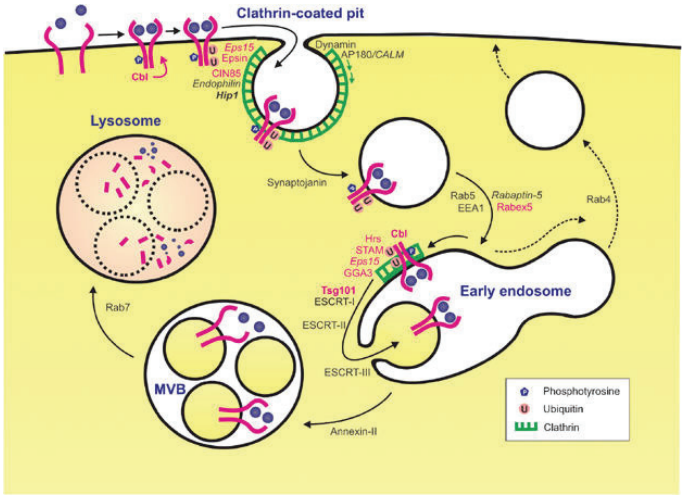

Figure 10. Endocytic downregulation of EGFR. Upon ligand binding and dimerization EGFR is ubiquitinated and internalized through clathrin-coated pits. After vesicle scission clathrin is released, and the vesicle fuses with the early endosome. Here the ubiquitinated receptor is believed to be retained from the recycling pathway by recruitment into flat clathrin-coated pits by Hrs. Retained receptor is then transferred through the three ESCRT complexes and internalized into inner vesicles on the early endosome, before being transported through the MVBs to the lysosome for degradation. The figure is from Bache *et al.*, 2004, and the figure legend is modified.

Ack1 in endocytosis

Activated Cdc42 associated kinase (Ack) is a nonreceptor protein tyrosine kinase. The human and murine forms of Ack are encoded by the TNK2 gene and are called Ack1. There are several isoforms of Ack1, isoform 1 being the best characterized. This isoform of Ack1 is 114 kDa. In this thesis Ack1 refers to isoform 1 of Ack1. Bovine Ack is called Ack2 and is encoded by the ACK2 gene. There are also several isoforms of bovine Ack, the best characterized isoform is 83 kDa. Throughout this thesis, Ack2 will refer to this isoform of bovine Ack.

Ack1 has been shown to be activated by EGF and to be recruited to EGFR following EGF stimulation (Galisteo *et al.*, 2006). The kinase was first identified by its binding to Cdc42 (Manser *et al.*, 1993) and contains an SH3 domain, a tyrosine kinase domain, a C-terminal proline-rich sequence, a Cdc42-binding CRIB domain and a Ralt homology

domain. The Ralt homology domain has been shown to mediate binding to the EGFR (Fiorentino *et al.*, 2000; Anastasi *et al.*, 2003; Shen *et al.*, 2006). Ack1 also contains a UBA domain, and this domain has been suggested to be important for EGFR degradation (Shen *et al.*, 2006). The activation of Ack1 following EGF stimulation has been shown to be dependent on Grb2 (Kato-Stankiewicz *et al.*, 2001). Ack1 is known to inhibit EGFR and TfR endocytosis by its ability to bind clathrin and to alter clathrin distribution (Teo *et al.*, 2001). Ack1 also localizes to clathrin-containing vesicles and co-localizes with both clathrin and AP-2. In addition, both Ack1 and Ack2 have been suggested to act as clathrin-assembly proteins regulated by Cdc42 (Yang *et al.*, 2001).

Ack1 specifically binds the GTP-bound form of Cdc42 (Manser *et al.*, 1993). Cdc42 is a member of the Rho family of GTPases and is known to be involved in regulation of the actin cytoskeleton, in regulation of cell polarity and has also been suggested to be involved in control of intracellular trafficking (reviewed in Cerione 2004). Ack2 has been shown to interact with SNX9, facilitating the degradation of the EGFR (Lin *et al.*, 2002). Recently, the interaction with SNX9 has also been shown for human Ack1 (Yeow-Fong *et al.*, 2005). The interaction between Ack1 and SNX9 occurs through the SH3 domain of SNX9 and the proline-rich sequences of Ack1, and SNX9 preferentially interacts with inactive Ack1 (Yeow-Fong *et al.*, 2005). In *Drosophila* the Ack1 orthologue DAck phosphorylates the SNX9 orthologue DSH3PX1, thereby causing decreased binding of SNX9 to WASP, an interaction that occurs through the SH3 domain of DSH3PX1 (Worby *et al.*, 2002). Hence, phosphorylation of SNX9 by Ack1 appears to alter the binding capacity of its SH3 domain. Additionally, in the presence of SNX9, Ack1 interacts with the endocytosis-linked protein synaptojanin-1 (Yeow-Fong *et al.*, 2005). It has also been shown that Ack1 phosphorylates WASP, thereby enhancing the ability of WASP to stimulate actin polymerization (Yokoyama *et al.*, 2005). N-WASP has been proposed to be important for efficient endocytosis of EGF and actin assembly at clathrin-coated pits (Benesch *et al.*, 2005).

Recently, it was shown that Ack1 in fact interacts with EGFR upon EGF stimulation, and that Ack1 is necessary for degradation of EGFR in a manner dependent on the UBA domain of Ack1 (Shen *et al.*, 2006).

AIMS OF THE STUDY

Endocytosis of the EGFR is important in regulation of receptor signaling. Internalization of the EGFR from the plasma membrane is essential for degradation, but the EGFR's kinase domain is cytosolic and in principle able to keep signaling until it is sorted into internal vesicles in early endosomes. Only then is the receptor destined for degradation in lysosomes.

Our first aim was to study the effect of different ubiquitination levels and patterns on endocytosis and endosomal degradation of the EGFR. Although it was known that Cbl can bind the EGFR both directly and indirectly, the reports on the effects of the interaction between the EGFR and Cbl on ubiquitination and internalization of the EGFR were contradictory (Waterman *et al.*, 2002; Jiang and Sorkin, 2003; Oksvold *et al.*, 2003; Shen *et al.*, 2006). By using PAE cells expressing wild type (wt) or Y1045F EGFR, we wanted to investigate whether direct and indirect binding of Cbl would give differences in ubiquitination, endocytosis and intracellular trafficking of the EGFR.

Our second aim was to characterize endosomal sorting of the EGFR. On endosomes, the EGFR is sorted either for recycling or to inner vesicles of MVBs for degradation (Gruenberg, 2001). The limiting membrane of early endosomes was known to contain functionally distinct microdomains (Raiborg *et al.*, 2001a). Some of these microdomains have coats, whereas others do not. We wanted to investigate the domains involved in sorting of the EGFR to lysosomes. Should there be different domains, we wanted to characterize the composition and function of these domains.

Our third aim was to characterize the function of Ack1. Ack1 had been shown to bind EGFR and clathrin, and overexpression had been shown to impair endocytosis of the TfR. We wanted to investigate whether Ack1 had a general function in endocytosis, or whether it had a specific function in endocytosis of the EGFR or endosomal sorting.

SUMMARY OF ARTICLES

Article I:

Direct interaction of Cbl with pTyr 1045 of the EGFR receptor (EGFR) is required to sort the EGFR to lysosomes for degradation.

In this article we showed that the Y1045F EGFR mutant, which does not bind Cbl directly, is ubiquitinated although not to the same extent as wild type EGFR. We showed that both direct and indirect binding of Cbl contributed to ubiquitination of the EGFR. Using immunofluorescence and confocal microscopy, we showed that the EGFR impaired in direct recruitment of Cbl through pTyr1045, was indeed internalized to early endosomes upon ligand activation. By immuno-EM we found that different interaction between EGFR and Cbl impacted on sorting on early endosomes and that lack of direct interaction resulted in inhibited sorting to the degradative pathway. The Y1045F EGFR mutant was not sorted into MVBs, but remained on the surrounding membrane of early endosomes, or it recycled back to the cell surface. We further demonstrate that the Y1045F EGFR mutant failed to co-localize with Hrs, a proposed adaptor for sorting of ubiquitinated cargo to the degradative pathway. By flow cytometry, we showed that the wt EGFR was downregulated upon EGF stimulation, whereas the Y1045F EGFR displayed impaired downregulation.

Article II:

Both clathrin-positive and -negative coats are involved in endosomal sorting of the EGF receptor.

In this study different microdomains present on sorting endosomes were characterized. We studied the localization of EGFR after ligand-induced internalization to early endosomes and observed that both Grb2 and Cbl localized to early endosomes upon internalization of the EGFR. Using immuno-EM, we demonstrated that Grb2 and Cbl together with the EGFR were sorted to coated microdomains on early endosomes and further transported into internal vesicles of MVBs. Using immunofluorescence and Rab5Q79L-induced enlarged endosomes we demonstrate that the microdomains to which

EGFR/Cbl/Grb2 were sorted, were only partially clathrin- and Hrs-positive. Immuno-EM further demonstrated that the sorted EGFR/Cbl/Grb2 only partially co-localized with Hrs and clathrin within coated domains on endosomes, and we propose that sorting of the EGFR involves both clathrin-positive and clathrin-negative coats.

Article III:

Over-expression of Ack1 inhibits internalization and endosomal sorting of the EGF receptor.

In this article we studied the function of Ack1 in endocytosis and intracellular sorting of the EGFR. We showed that Ack1 co-localized with EGF on EEA1-positive early endosomes. Using immuno-EM, we studied the intracellular localization of Ack1 and confirmed that Ack1 localized to early endosome-like compartments as well as to a reticulum consisting of interconnected coated tubules. We showed that endocytosed EGF co-localized with Ack1 on early endosomes and on the Ack1 positive reticulum and that overexpression of Ack1 inhibited internalization of EGFR. We further demonstrated that internalized EGFR was retained in early endosomes and that translocation into MVBs was inhibited. These results led us to propose that Ack1 is involved in endosomal sorting of the EGFR. Also, we confirmed that overexpression of Ack1 caused sequestration of clathrin intracellularly and thereby inhibited clathrin-mediated endocytosis. Furthermore, we found that overexpression of Ack1 induced sequestration of dynamin, and probably the inhibited clathrin-independent endocytosis could be explained by this.

METHODOLOGICAL CONSIDERATIONS

Experimental models

Cultured cell lines are powerful tools in molecular biology and are important for studies of cell biology. They allow differences in protein expression by transient or stable transfection and studies of protein localization and protein-protein interactions upon different manipulations. As cell lines can be cultured for longer periods of time, cell lines allow multiple experiments to be performed. Genotypic and phenotypic alterations may, however, occur over time. To limit such events, cells were kept in culture for no more than 8 weeks. Cells with low passage numbers were stored in liquid nitrogen. When using cultured cell lines, one should, however, be careful not to generalize observations made in one specific cell line. To enable culturing, cells must be immortalized, and different cell lines have different origins in addition to being differentiated to various degrees. It is therefore important not to immediately generalize importance of observations made in one cell line. Also, it is important to be aware of differing subcellular protein compositions in specific cell lines. Differences in the speed of cellular processes like endocytosis could also be cell specific.

In this work three different cell lines have been used. The human cervical carcinoma cell line HeLa and the human laryngeal carcinoma cell line Hep2 both express relatively high amounts of EGFR. Whereas the HeLa cells express approximately $7*10^4$ EGFRs at the plasma membrane (Ringerike *et al.*, 1998), we estimated the amount of EGFR on the plasma membrane of Hep2 cells to be roughly $5*10^5$ by flow cytometry. The morphology of the cells is useful for microscopy studies as the ratio of cytosol to nucleus is high, allowing studies of cytosolic proteins. The studies on the Y1045F EGFR mutant were mostly performed in cells derived from a Porcine Aortic Endothelial (PAE) cell. The original PAE cells do not express endogenous EGFR, but cells stably transfected with either human wt EGFR or Y1045F EGFR have been made (Jiang and Sorkin, 2003). The different cell lines expressed relatively similar amounts of the EGFR, the amount of Y1045F EGFR being slightly higher than that of wt EGFR. One should be aware that these cells are of porcine origin, and that EGFR of human origin may not bind all

proteins of porcine origin as efficiently the homologous proteins in cells of human origin. The cell line is well described, however, and the EGFR appears to be endocytosed in a manner similar to that of endogenous EGFR in human cells (Carter and Sorkin, 1998).

Immunological methods

Another powerful tool in cell biology is the different immunological methods. These methods are based on the very specific recognition between an antigenic epitope and an antibody. These methods are useful for visualization of specific proteins and their localization in cells using microscopy, and also for detecting and quantifying specific proteins in total cell lysate, after lysis of the cell. Another important method is the use of antibodies for immunoprecipitation studies, both to study protein modifications and protein-protein interactions. Using immunological methods, the critical point is having good antibodies. They must be specific for the protein in question and bind with high affinity. Antibodies with low specificity may give false positives, and misinterpretation of data. All our antibodies were analyzed for specificity.

Microscopy

Immunostaining combined with confocal and electron microscopy enabled us to study the intracellular localization of different proteins. Using multiple labeling for immunofluorescence microscopy, bleedthrough from the different fluorochromes can cause false interpretations of signals, a problem we tried to minimize by taking images sequentially. Also, when using multiple antibodies simultaneous, cross-reaction between antibodies may occur, giving rise to erroneous interpretations. It is therefore important to include negative controls when doing double or triple labeling experiments. We also tried to minimize nonspecific binding by incubating the prepared cells with BSA prior to labeling. By using fluorescently labeled EGF, we avoid problems connected to antibody cross-reaction and non-specificity when studying localization of the EGF/EGFR-complex. One must be aware, however, that EGF may dissociate from the EGFR, so that fluorescent EGF may not always co-localize with the EGFR. Using fluorescently labeled EGF can also be an advantage when studying receptor endocytosis, as only newly endocytosed EGFR will be detected and not newly synthesized intracellular EGFRs being

transported towards the plasma membrane. The resolution of confocal microscopy is limited, and immuno-EM is a better tool for studying protein localization to specific membrane domains. Another advantage of using EM is that electron-dense membranes and coats can be visualized without specific markers. A limitation when using both confocal microscopy and EM, is that the images obtained are only 2-dimensional. Using confocal microscopy, this can lead to false positive co-localization of proteins that are in fact separated in the third dimension. In case of EM, the problem with 2-dimensional pictures is often that the section is so thin that important information can be lost. Continuous structures may be difficult to follow, and may be misinterpreted as two separate structures if a part of the structure lies outside the slide. Immuno-EM also allows quantitation of the labeling, but like other immunolabeling techniques, one must be aware of limitations such as antigen accessibility, labeling efficiency and cross-reactivity.

Internalization of EGF

We measured internalization, recycling, and degradation of EGFR by incubating cells with ^{125}I-EGF and then measuring the radioactivity in different fractions of cells and medium. This method allows for highly reproducible and quantitative data, obtained from much larger total amount of cells than when using microscopy. One must keep in mind, however, that one is in fact studying the internalization of EGF, and not the total pool of the EGFR. As for fluorescent EGF in microscopy studies, ^{125}I-EGF may dissociate from the EGFR, so there may be discrepancies between the observed localization and degradation of EGF, and that of the EGFR. EGFR degradation can, however, be readily confirmed by Western blotting of total cell lysate.

Enlarged endosomes by Rab5Q79L overexpression

The confocal microscopy resolution limits the studies of small microdomains on endosomes. To solve this problem we overexpressed the constitutively active mutant of Rab5, Rab5Q79L, in HeLa cells. This causes the formation of enlarged early endosomes in the cells due to increased fusion of early endosomes (Stenmark *et al.*, 1994). The introduction of enlarged endosomes allows studies of microdomains on early endosomes even with confocal microscopy. The endosomal microdomains formed upon Rab5Q79L

overexpression have been well characterized, and the presence of distinct microdomains has been confirmed by other methods as well. It is important, however, to be aware of possible sources of misinterpretation when using this method. First, small vesicles docking at the early endosome may be misinterpreted as endosomal microdomains. This is only avoided with experience in studying enlarged endosomes. Also, the induction of large endosomes causes redistribution of lysosomal markers and appears to disrupt lysosome biogenesis (Rosenfeld *et al.*, 2001).

Transient overexpression of Ack1

Transient overexpression of proteins is a widely used and powerful tool in molecular cell biology. The method allows easier detection of interactions with target proteins and studies of localization. Overexpression of fluorescently tagged protein is helpful in studying protein localization by microscopy. Increased expression of a specific protein may also result in altered cell phenotype and may thereby indicate the function of the endogenous protein. Overexpression of proteins, with or without small tags such as HA, is helpful when endogenous expression of the protein is too low to allow microscopic studies. Tags may be useful to overcome problems such as lack of specific antibodies, because antibodies to the tags are normally easier obtainable than antibodies to a specific protein. An important thing to consider when overexpressing proteins is that tightly controlled interactions with other proteins may be disturbed. Also, interactions that normally rarely occur in the cell may be induced by overexpression of proteins. Interactions observed when overexpressing a protein may thus not take place at normal expression levels. Also, interacting proteins that normally participate in multiple reactions in the cell may be sequestered upon overexpression of one of its interaction partners and thus induce false phenotypes. This is the case with overexpression of Ack1. Expressed at high levels, Ack1 sequesters clathrin in the cell, thereby inhibiting clathrin-mediated endocytosis.

DISCUSSION OF RESULTS

Ubiquitination and endocytosis

There is currently agreement that ubiquitination is important in endocytic downregulation of the EGFR. Exactly which step of receptor endocytosis is controlled by ubiquitination is, however, still debated. Because of conflicting evidence, the importance of ubiquitination in initial endocytosis of the EGFR and in sorting on early endosomes is not fully understood. We used the EGFR mutant Y1045F for this purpose, as Cbl, the ubiquitin ligase responsible for ubiquitination of the EGFR, does not bind directly to this receptor (Levkowitz *et al.*, 1998; Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999; Waterman *et al.*, 1999; Yokouchi *et al.*, 1999; Lill *et al.*, 2000). We found that the Y1045F EGFR was not degraded. This is consistent with the results of others demonstrating that direct binding of Cbl to the EGFR is necessary for EGFR degradation (Levkowitz *et al.*, 1998; Levkowitz *et al.*, 1999; Waterman *et al.*, 1999; Jiang and Sorkin, 2003). Together with the observed arrest of Y1045F EGFR at the limiting membrane of early endosomes, this supports the notion that ubiquitination is important in sorting the EGFR to MVBs and lysosomes for degradation (Levkowitz *et al.*, 1999; Longva *et al.*, 2002; Duan *et al.*, 2003).

To examine this further, we studied ubiquitination of the EGFR and found the Y1045F EGFR to be ubiquitinated to some extent. This was in agreement with results reported by Waterman *et al*. (Waterman *et al.*, 2002) who showed that Y1045F EGFR was ubiquitinated, especially upon overexpression of Cbl and Grb2. Our results were, however, in contrast to other publications showing lack of Y1045F EGFR ubiquitination (Levkowitz *et al.*, 1999; Jiang and Sorkin, 2003). A recent study employing tandem mass spectrometry confirmed that the Y1045F EGFR mutant is indeed ubiquitinated upon stimulation with EGF. Out of 6 lysine residues identified as sites of ubiquitination within the kinase domain of EGFR, 2 were still ubiquitinated in the Y1045F EGFR mutant (Huang *et al.*, 2006). Thus, ubiquitination is still a possible signal for internalization of the EGFR by clathrin-mediated endocytosis.

Ubiquitination of the EGFR has been shown to occur at the plasma membrane (Stang *et al.*, 2000), and Cbl has been demonstrated to be important for internalization of the EGFR (Thien *et al.*, 2001; Soubeyran *et al.*, 2002; Waterman *et al.*, 2002; Jiang and Sorkin, 2003; Huang and Sorkin, 2005). We have found that ubiquitination of the EGFR is necessary for its recruitment into coated pits (Stang *et al.*, 2004). It has been found that ubiquitination of the EGFR by Cbl can occur both via the direct interaction between the EGFR and Cbl and via indirect interactions through Grb2 (Waterman *et al.*, 2002). In paper I we confirmed this by overexpression of Cbl- and Grb2 mutants. It has been proposed that these two interactions represent different, independent pathways for receptor ubiquitination (Waterman *et al.*, 2002). Others have recently proposed that Grb2 mediated recruitment of Cbl to the EGFR is necessary for internalization, whereas the direct interaction is necessary for sorting of the EGFR in early endosomes (Huang and Sorkin, 2005). These results are in agreement with ours.

It has been proposed that the internalization apparatus for ubiquitinated proteins at the plasma membrane requires only minimal ubiquitination of receptors for interaction, whereas the sorting apparatus for ubiquitinated cargo at the endosome requires more ubiquitination for binding (Huang *et al.*, 2006). This is not contradictory to our results. Another explanation, however, for the different requirements in interactions between Cbl and EGFR in internalization and endosomal sorting, could be formation of different ubiquitin patterns by the different interactions. Support for this model lies in the recent study of UIMs of ubiquitin binding proteins. The UIM domain of epsin 1, an adaptor protein suggested to function in clathrin-mediated endocytosis of ubiquitinated cargo, consists of multiple tandem UIMs that preferentially bind polyubiquitin (Hawryluk *et al.*, 2006). Hrs, which is responsible for endosomal sorting of ubiquitinated cargo, has only one UIM. This UIM actually binds two molecules of ubiquitin simultaneously, each on different sides of an α-helix (Hirano *et al.*, 2006). The differences in binding of ubiquitin to UIMs of epsin 1 and Hrs support the notion that different ubiquitin patterns are important at different stages of endocytosis and intracellular sorting. In support of this we found the Y1045F EGFR mutant, although ubiquitinated, to be excluded from Hrs-

positive coats on early endosomes. Wt EGFR, however, was concentrated in Hrs-positive coats on the limiting membrane of early endosomes.

In paper II, using immuno-EM, we found that also Cbl and Grb2 are recruited into coated microdomains on endosomes. This is in agreement with previous studies, demonstrating recruitment of Cbl and Grb2 to early endosomes upon EGF stimulation (Sorkin *et al.*, 2000; Jiang and Sorkin, 2002; Longva *et al.*, 2002; Huang and Sorkin, 2005). This further supports the notion that sustained ubiquitination of the EGFR is necessary for endosomal sorting of the EGFR for degradation by the lysosomal pathway (Longva *et al.*, 2002). Although the importance of Grb2 for efficient endosomal sorting is not yet fully understood, it has been proposed that on the plasma membrane, Grb2 functions in receptor endocytosis by recruiting Cbl (Huang and Sorkin, 2005). Also, Grb2 appears to be important for efficient recruitment of Cbl to early endosomes (Jiang and Sorkin, 2003). Thus, our results further support the notion that sorting of the EGFR for degradation relies on sustained ubiquitination of the receptor and on interaction with the sorting machinery consisting of Hrs/STAM and the ESCRT complexes (reviewed in Raiborg *et al.*, 2003).

The finding that EGFR localizes to Hrs/clathrin-positive coats is in agreement with previous immunofluorescence and EM studies (Raiborg *et al.*, 2002; Sachse *et al.*, 2002). It has been demonstrated that endosomes contain multiple coated domains (Murk *et al.*, 2003), and both Hrs-positive and Hrs-negative coats have been observed (Sachse *et al.*, 2004). We found the EGFR/Cbl/Grb2 complexes also in coated microdomains negative for Hrs and clathrin. Clathrin-negative coats have been reported by others (Sachse *et al.*, 2004), but the exact function and composition of these coats are unknown. It is possible that ESCRT complexes also form coats on early endosomes, and our observation would then be in agreement with the findings that ubiquitinated cargo is transferred from Hrs/STAM complexes to ESCRT complexes before being sorted into internal vesicles on MVBs (reviewed in Raiborg *et al.*, 2003). The late stages of sorting would then involve coats devoid of Hrs and clathrin. This is supported by the recent findings that the Hrs/clathrin-coated microdomains are dynamic structures where Hrs and clathrin are

exchanged with similar kinetics. A model is proposed in which clathrin and Hrs are released from the particular microdomains simultaneously, allowing recruitment of the ESCRT complexes before sorting into internal vesicles (Raiborg *et al.*, 2006). Thus, it is possible that the coats devoid of clathrin and Hrs represent EGFR in complex with the ESCRTs.

Ack1 and EGFR endocytosis

We found that overexpression of Ack1 sequestered clathrin and inhibited internalization of the EGFR. This is in agreement with previous studies, demonstrating that overexpression of Ack1 or Ack2 perturbs clathrin distribution and impairs TfR internalization (Teo *et al.*, 2001; Yang *et al.*, 2001). As internalization of the EGFR is also strictly clathrin-dependent (Kazazic *et al.*, 2006), the observation that overexpression of Ack1 also impairs EGF endocytosis is not surprising. It must be emphasized, however, that this is an effect of overexpression of Ack1, and probably not the effect of endogenous Ack1 *in vivo*. Others have observed, however, that overexpression of Ack2 together with SNX9 enhances internalization of the EGFR (Lin *et al.*, 2002). Thus, it is likely that the effect of transient expression of Ack1 is very sensitive to expression levels achieved. The fact that Ack1 was localized to early endosomes together with EGF, and that overexpression retained the EGFR on the limiting membrane of early endosomes, suggests the involvement of Ack1 in EGFR sorting. Involvement of Ack1 in EGFR sorting is also suggested in the recent study, where siRNA-induced downregulation of Ack1 was demonstrated to affect downregulation of the EGFR (Shen *et al.*, 2006). However, Shen *et al*. suggested that over-expression of Ack1 facilitated downregulation of the EGFR. The contradicting conclusions in their and our studies could partially be explained by sequestration of proteins involved in sorting of the EGFR upon overexpression of Ack1.

We further found that overexpression of Ack1 in addition to inhibiting clathrin-dependent endocytosis inhibited the clathrin-independent endocytosis of MHC-I. The inhibited endocytosis of MHC-I is most likely due to an Ack1-induced sequestration of dynamin, as we found that dynamin co-localized with Ack1 intracellularly. Altogether, these results

show that overexpression of Ack1 can interfere with endocytosis and thereby with signal-transduction at several stages. This can again interfere with growth regulation.

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