Chapter 12

Mutual Regulation of Receptor-Mediated Cell Signalling and Endocytosis: EGF Receptor System as an Example

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Additional information is available at the end of the chapter

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1. Introduction

Epidermal growth factor (EGF) receptor (EGFR), also known as HER1 or ErbB1, is the prototypal member of the superfamily of receptors with intrinsic tyrosine kinase activity and is widely expressed in many cell types including epithelial and mesenchymal lineages [1, 2]. The other three members of the ErbB receptor family include Her2/ErbB2/neu [3, 4], Her3/ErbB3 [5] and Her4/ErbB4 [6] (Fig. 1A). EGFR is a 170 kDa membrane glycoprotein composed of three domains. The heavily glycosylated 622-amino acid extracellular domain containing two cysteine rich regions is responsible for ligand binding. The transmembrane domain is a single 23-amino acid α-helical transmembrane peptide. The 542-residue intracellular cytoplasmic domain contains a 250-amino acid conserved protein tyrosine kinase core followed by a 229-amino acid C-terminal tail with regulatory tyrosine residues (Fig. 1B) [7]. Eleven ligands have been identified for ErbB receptors. These ligands can be classified into three groups based on their ability to bind to different ErbB receptors. The first group of ligands includes EGF, transforming growth factor-α, amphiregulin and epgen, which specifically binds to EGFR. The second group of ligands includes betacellulin, heparin-binding EGF and epiregulin, which binds to both EGFR and ErbB4. The third group of ligands includes neuregulin/heregulin, which binds to ErbB3 and ErbB4 [8, 9] (Fig. 1A).

The EGFR family of receptor tyrosine kinases lies at the head of a complex signal transduction cascade that modulates cell proliferation, survival, adhesion, migration and differentiation [1, 2, 10]. While growth factor-induced EGFR signalling is essential for many normal morphogenic processes and is involved in numerous additional cellular responses, the aberrant activity of the members of this receptor family has been shown to play a key role in the development and growth of tumour cells [10-12]. The ErbB receptors were first implicated in cancer when the avian erythroblastosis tumor virus was found to encode an

aberrant form of EGFR. Now, EGFR has been implicated in many cancers including squamous cell head and neck cancer, colorectal cancer, non-small cell lung cancer, gastric cancer, pancreatic cancer, breast cancer, ovarian cancer, renal cancer, glimas prostatic cancer and cervical cancer [13]. The dysregulation of ErbB receptor signalling in cancer can occur by various mechanisms, including overexpression due to gene amplification, autocrine ligand production, heterodimerization, deficiency in endocytosis, and gene mutations that increase receptor transcription, translation, protein stability and kinase activity[14, 15].

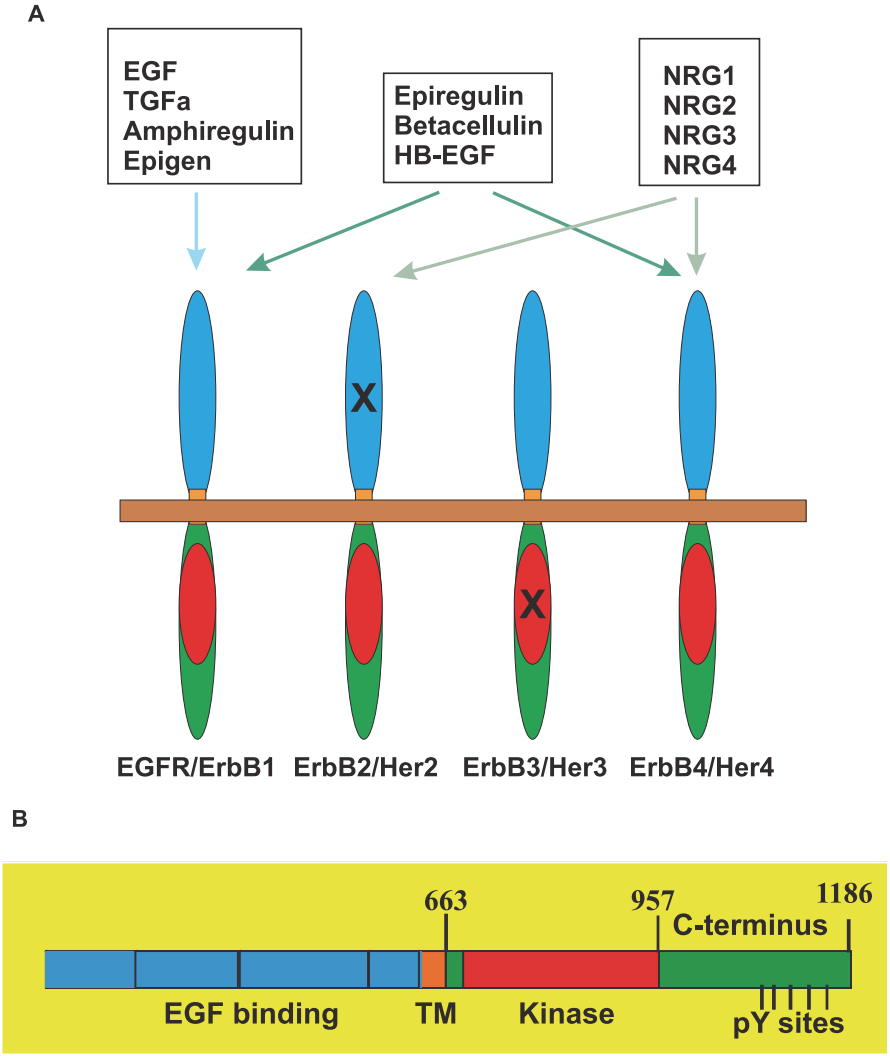

Figure 1. ErbB receptor family. (A) ErbB receptors and their ligands. ErbB family receptors are composed of four members: EGFR/ErbB1/Her1, ErbB2/Her2/neu, ErbB3/Her3 and ErbB4/Her4. Eleven ligands are identified for ErbB family receptors. (B) Linear structure of EGFR.

The binding of EGF at the cell surface induces dimerization of EGFR, which results in the activation of EGFR tyrosine kinase and receptor trans-autophosphorylation [16, 17]. EGFR activation stimulates various signaling pathways, leading to cell mitogenesis and survival [9, 10]. EGFR is overexpressed or hyper activated in many epithelial tumors and plays important roles in cancer development and progression [12]. The activated receptors are rapidly internalized into endosomes and eventually degraded in lysosomes [18]. Initially, the endocytosis of ligand-activated receptors was considered a mechanism to attenuate signaling. Recently, more evidence suggests that the internalized receptors may maintain their ability to generate cell signaling in endosomes [19-23]. Thus, the alteration of EGFR endocytosis may result in abnormal cell signaling, leading to cancer. On the other hand, EGFR endocytosis is firmly regulated by signal recognition and various signaling proteins at every step.

2. EGFR-mediated cell signaling

EGFR plays important roles in initiating cell signaling to produce specific effects on cell growth and development [9, 10]. EGFR is activated through the homodimerization or heterodimerization with other ErbBs such as ErbB2 and ErbB3 in response to ligand stimulation (Fig. 2)[2]. The dimerization of EGFR at the plasma membrane induces the activation of the EGFR tyrosine kinase and trans-autophosphorylation. The sites of tyrosine phosphorylation in the activated EGFR form signaling complexes with many signaling proteins, including Grb2, Shc, phospholipase C-γ1 (PLC-γ1), the p85α subunit of PI3K (p85), p120 rasGAP, Src, Stats, and Cbl [2, 24-26] [2]. The formation of the receptor-signaling protein complexes then initiates the activation of various signaling pathways (Fig. 3A)[9-11, 27-29].

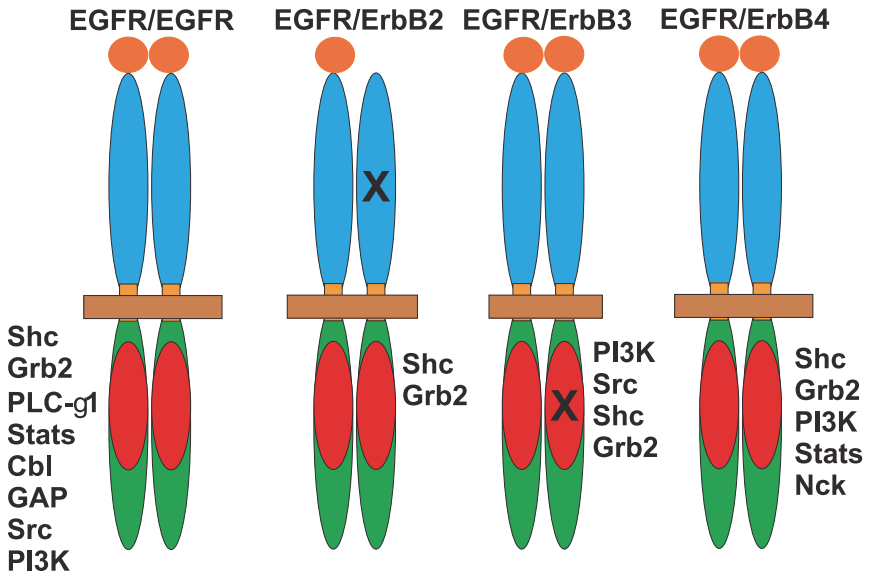

Figure 2. Dimerization of EGFR and the association with signaling proteins. EGFR is homodimerized or heterodimerized with other ErbB proteins in response to ligand.

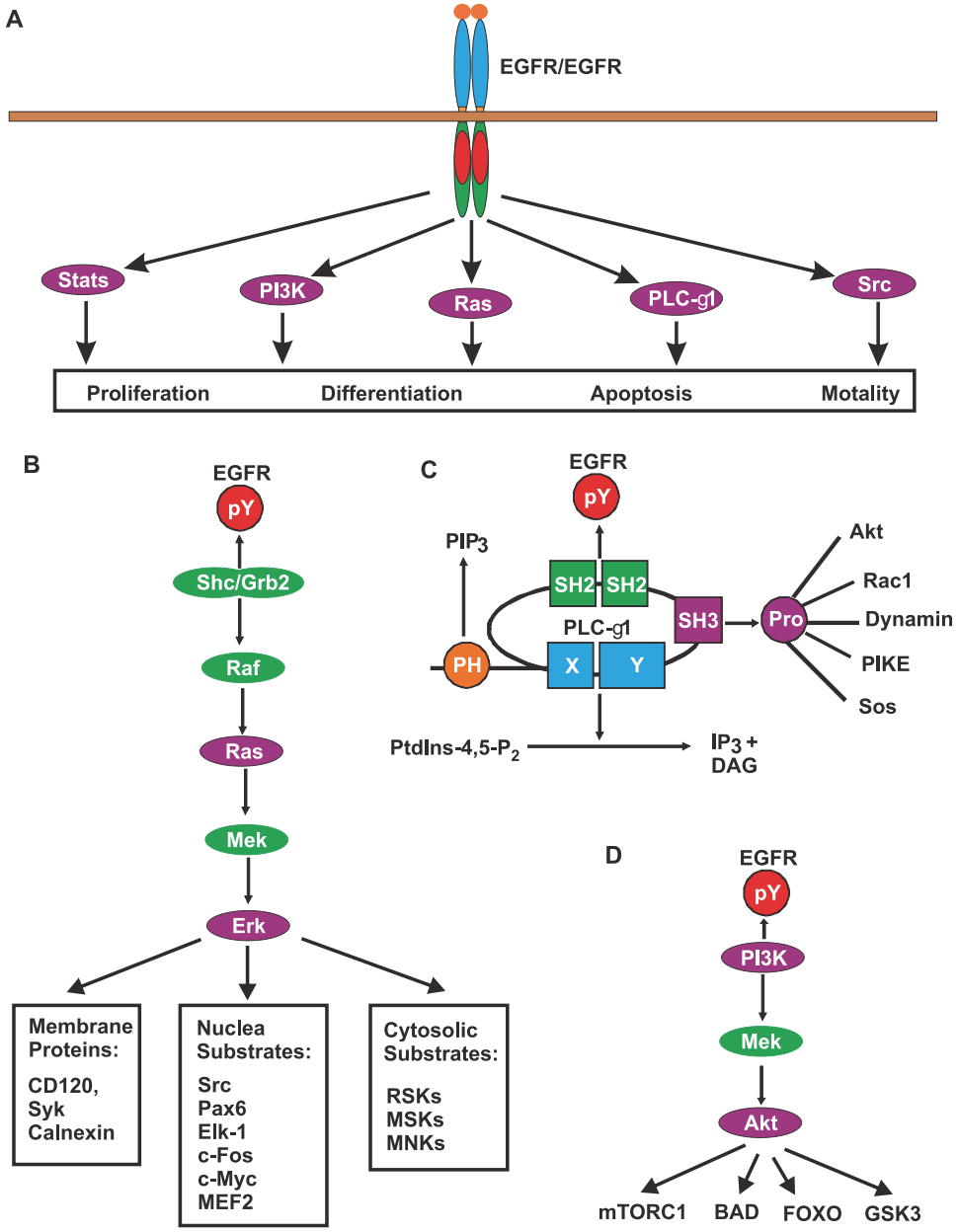

Figure 3. Signaling pathways activated by EGFR. (A) Binding of EGF to EGFR at the plasma membrane initiate the activation of various signaling pathways. The well-defined pathways include Ras-Erk pathway, PI3K-Akt pathway, PLC-γ1 pathway, Stat pathway and Src pathway. (B)The signaling cascade of Ras-Erk pathway. (C) The signaling cascade of PLC-γ1 pathway. (D) The signaling cascade of PI3K-Akt pathway.

The activated EGFR interacts with Shc and Grb2 through multiple phosphorylated tyrosine (pY) residues localized at the C-terminus, which results in the recruitment of Sos to the plasma membrane to activate Ras. Activated Ras mediates Raf activation, which then phosphorylates and activates MEK. Activated MEK then phosphorylates and activates ERK. Activated ERK phosphorylates Rsk, which in turn translocates into the nucleus to activate transcription factors such as c-fos and SRF. Activated ERK may also translocate into the nucleus to activate transcription factors such as Elk1 and c-fos, which is critical in controlling cell mitogenesis (Fig. 3B) [2, 24, 30-34].

Activated EGFR also interacts with PLC-γ1 with multiple pY residues at the C-terminal regulatory domain, which results in the phosphorylation of PLC-γ1 and an increase in its enzymatic activity [35-37]. Active PLC-γ1 hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)P_2) to form the second messengers inositol 1, 4, 5-triphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ and DAG mobilize intracellular calcium and activate protein kinase C (PKC), respectively. Recent studies have shown that PLC-γ1 is involved in broad cell signaling. Interestingly, most recently identified interactions between PLC-γ1 and its binding proteins are mediated by its SH3 domain. EGF stimulates the interaction between PLC-γ1 and PLD_2, which is mediated by the PLC-γ1 SH3 domain [38]. PLC-γ1 binds directly to Akt in response to EGF through its SH3 domain [39]. The PLC-γ1 SH3 domain acts as a guanine nucleotide exchange factor (GEF) for PIKE [40], dynamin [41] and Rac1 [42]. The activated PLC-γ1 regulates cell mitogenesis and migration (Fig. 3C)[39, 42-44].

Activated EGFR also activates PI3K either through its direct interaction with the p85α subunit or through the activated Ras [45, 46]. Activated PI3K then catalyzes the production of the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3) by phosphorylating phosphatidylinositol-4,5-bisphosphate (PIP_2). A direct antagonist of PI3K is the phosphatase and tensin homologue deleted on chromosome 10 (PTEN). PTEN dephosphorylates PIP_3 into PIP_2 to reverse the activity of PI3K and therefore function as an important negative controlling element of incoming signals. PIP_3 transduces activating signals by binding to pleckstrin homology (PH) domains of proteins to recruit them to the cell membrane. One centrally important downstream mediator of the PI3K signalling cascade is the serine threonine (Thr) kinase Akt. Akt is recruited to the plasma membrane by its SH3 domain interaction with PIP3, which exposes Akt Thr 308 for phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK-1), which is already located at the membrane. The rapamycin complex 2 (mTORC2) phosphorylates Ser 473 in the C-terminus, which leads to full Akt activation. Activated Akt then mediates signals promoting cellular growth and survival and suppresses pro-apoptotic signals. Akt phosphorylates several intracellular proteins, including forkhead box O transcription factors (FoxO), the BCL2-associated agonist of cell death (BAD), and the glycogen synthase kinase 3 (GSK3), to promote cell cycle entry and cell survival. The proteins TSC1 (Hamartin) and TSC2 (Tuberin) form a complex that inhibits the activity of the small G-protein ras homologue enriched in the brain (Rheb), which is necessary for mTORC1 activation. The Akt-mediated phosphorylation of TSC2 releases Rheb from its inhibited state. Rheb then accumulates in a GTP-bound state

and can directly activate mTORC1, which phosphorylates the p70S6 kinase (S6K1) and the eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), leading to increased protein translation (Fig. 3D), which protects the cell from undergoing apoptosis [45, 47, 48].

Activated EGFR also activate Stats directly by binding to and phosphorylating Stats, or indirectly by activating c-Src. Activation can occur via cytokine signaling (IL-6), growth factor receptor signaling (EGFR), or non-receptor tyrosine kinase signaling (Src). JAK is not required when Stats bind directly to EGFR for activation, but JAK provides maximal activation of Stats phosphorylated by EGFR-activated Src. Grb2 and SOCS can inhibit Stat-mediated EGFR signaling respectively, by either binding to the STAT activation site on EGFR or by binding to JAK to suppress Src activation of Stats. Once activated, Stats dimerize and translocate to the nucleus where they activate the transcription of genes involved in proliferation, differentiation, and survival [49].

Importantly, Src kinases, which have been reported to be activated in many cancers with high EGFR levels, have been shown to potentiate EGFR signaling [50-52]. The c-Src potentiation of EGFR has been demonstrated to be associated with the c-Src-dependent phosphorylation of EGFR and the complex formation between c-Src and EGFR [50, 51]. In addition to focal adhesion kinase (FAK), which is involved in the regulation of adhesion and migration, PI3K and Stat3 are also substrates for c-Src [53]. Although the Src kinase has been linked with the development and progression of cancer for many years, we still do not completely understand its role in cancer [54]. Src is a member of a ten-gene family (FYN, YES, BLK,FRK, FGR, HCK, LCK LYN, and SRMS) of non-RTKs that play a fundamental role in the regulation of cell proliferation, migration, adhesion, and tumor angiogenesis [55, 56]. Src signaling is cross-connected with many signaling pathways, such as the PI3K and Stat pathway [55, 56]. Even though tyrosine kinase activity of Src is independent of RTK signaling, it may interact with RTKs such as EGFR. As such, Src-EGFR interaction may enhance EGFR signaling, and on the other hand it may be involved in resistance to EGFR-targeted therapy [54, 57].

3. EGFR-mediated endocytosis

3.1. Clathrin-dependent and clathrin-independent endocytic pathways

The concept of receptor-mediated endocytosis was formulated in 1974 to explain how the sequential cell surface binding, internalization, and intracellular degradation of plasma low density lipoprotein (LDL) regulates cellular cholesterol metabolism [58]. Receptor-mediated endocytosis is a multiple step event [58]. In general, receptor-mediated endocytosis consists of two stages: internalization and intracellular trafficking. Endocytic pathways are generally classified as either clathrin-dependent or clathrin-independent. Much work has focused on clathrin-mediated endocytosis (CME). In this process, cargo proteins are recruited into developing clathrin-coated pits (CCPs), and subsequently form clathrin-coated vesicles (CCVs) [59]. Several proteins or protein complexes, including clathrin, adaptin AP-2, dynamin and Eps15, participate in the CME of all receptors. Adaptin AP-2 is a cytoplasmic protein complex that interacts with the cytoplasmic tails of various receptors. These

interactions are thought to account for the ability of cells to selectively direct receptors to CCVs. Clathrin is ideally designed to form a scaffold which, when attached to the membrane, causes the membrane to deform into a budding vesicle. Clathrin presumably binds to the membrane by interacting with membrane-bound AP-2. Dynamin has been identified as a major player in the endocytic pathway and is essential for the scission of coated vesicles. Eps15 is an essential component of the early endocytic pathway [59-61].

Although CME is certainly an extremely important endocytic mechanism, accounting for a large proportion of endocytic events, an ever expanding array of cargos has been shown to undergo non-clathrin-mediated endocytosis (NCE) [62]. Many NCE pathways have been reported, including caveolar-type endocytosis, CLIC/GEEC-type endocytosis, the putative flotillin-associated endocytic structures, phagocytosis, macropinocytosis, dorsal ruffles (or waves), and entosis [62, 63]. Caveolar-type endocytosis is the best studied NCE.

3.2. Endocytic and sorting signals

The targeting of transmembrane proteins to different compartments of the endocytic pathways is largely dependent upon sorting signals contained within the cytoplasmic domains of the proteins [64-66]. Most of these sorting signals are short, linear sequences of amino acid residues. These signals can be classified to two groups. One group of signals is referred to as tyrosine-based sorting signals and the other group of signals is known as dileucine-based signals. All of these signals are recognized by components of protein coats peripherally associated with the cytosolic face of membranes [66].

Tyrosine-based signals constitute a family of degenerate motifs minimally defined by the presence of a critical tyrosine residue [66]. Most tyrosine-based signals conform to the consensus motifs YXXΦ (Y is tyrosine, X is any amino acid and Φ is an amino acid with a bulky hydrophobic side chain) [67] or NPXY (N is asparagine and P is proline) [68-71]. It was shown by several groups that the substitution of tyrosine residues in the cytosolic domains of various endocytic receptors devoid of NPXY motifs impaired internalization [72-77]. NPXY signals have been shown to mediate only the rapid internalization of a subset of type I integral membrane proteins, and not mediate other intracellular sorting events. The interaction between NPXY motif and endocytic protein is less understood. However, several proteins, including clathrin, AP-2, and Dab2, have been proposed to function as recognition proteins for NPXY signals. There are several NPXY motifs located in the EGFR C-terminus [78]. Systematic mutational analyses led to the identification of another tyrosine-based motif, YXXΦ, as the major determinant of endocytosis of the mannose 6-phosphate as well as many other transmembrane proteins [67, 78, 79]. In mammalian cells, virtually all YXXΦ signals mediate rapid internalization from the cell surface. Some YXXΦ signals can additionally mediate lysosomal targeting [64-66]. Recent evidence suggests that the μ2 subunit of AP2 directly interacts with YXXΦ to mediate rapid internalization [80-83].

Di-leucine-based sorting signals have been implicated in various sorting process [78]. Two classes of di-leucine-based sorting motifs have been distinguished. [DE]XXXL[LI] signals play critical roles in the sorting of many type I, type II and multispanning transmembrane

proteins. The [DE]XXXL[LI] signals in mammalian proteins mediate rapid internalization and target the proteins to endosomal-lysosomal compartments, suggesting that they can be recognized both at the plasma membrane and at intracellular locations. [DE]XXXL[LI] signals are recognized by the adaptor protein (AP) complexes. DXXLL signals are present in several transmembrane receptors and other proteins that cycle between the TGN and endosomes. DXXLL signals are recognized by another family of adaptors known as GGAs.

Ubiquitination of cytosolic lysine residues constitutes another important signal for sorting transmembrane receptors at various stages of the endosomal-lysosomal system. Ubiquitin is a globular protein consisting of 76 amino acids that is able to covalently conjugate to other proteins [84]. Ubiquitin is covalently conjugated to proteins by forming a bond between the carboxy-terminal glycine of ubiquitin and the ε-NH_2 group of a lysine residue in the substrate protein. Alternatively, ubiquitin can be conjugated to the α-NH_2 group of the N-terminal amino acid of the substrate [85, 86]. Conjugated ubiquitin is recognized by UIM, UBA, or UBC domains present within many components of the internalization and lysosomal targeting machinery. It has been shown that EGFR is ubiquitinated in response to EGF, which plays an important role in EGFR degradation in lysosomes. The presence of these various type of sorting signals within the transmembrane receptors and their interaction with the signal recognition proteins ensures the dynamic but accurate distribution of transmembrane proteins to different compartments along the endocytic pathways.

3.3. Endocytosis of EGFR

The first comprehensive study of EGF endocytosis, in which many of the key concepts of internalization and lysosomal degradation of EGF have been established, was published by Carpenter and Cohen [87]. The binding of EGF results in the clustering and internalization of EGFR. The accumulation of EGF and EGFR can be detected in the early endosome after 1-5 min of incubation with EGF at 37°C. EGF and EGFR accumulate in late endosomes after 10-20 min at 37°C. A substantial number of EGFR can be detected in organelles with typical biochemical and morphological features of mature lysosomes only after 40-60 min of continuous internalization at 37°C [16, 88]. Intracellular trafficking of receptors involves a series of membrane budding and fusion events [89]. Endosome fusion is regulated by specific cytosolic and membrane-associated protein factors, including a group of Ras-like small guanosine triphosphatases (GTPases) called Rabs [90-92]. Four classes of endocytic organelles are typically distinguished based largely on their relative kinetics of labeling by endocytic tracers: early endosomes (EEs), late endosomes (LEs), recycling vesicles (RVs), and lysosomes [65]. The precise relationship among these structures has yet to be determined, and in fact may never be known because of the great plasticity and dynamics of the system.

The internalization of constitutively internalized receptors is largely mediated by sorting signals such as YXXΦ and NPXY. However, for the receptors that are internalized in response to ligand binding, there is likely some means of switching their sorting signals on and off [93]. Given that ligand binding is essential for the rapid internalization of EGFR, the

events induced by the ligand binding likely contribute to the regulation of ligand-induced EGFR internalization. These events include receptor dimerization, activation of intrinsic tyrosine kinase activity, autophosphorylation and association with various binding proteins.

The initial results are very controversial regarding the role of EGFR kinase activity in EGFR internalization. Data from some research groups suggest that kinase-dead EGFR is deficient in EGF-induced internalization [94, 95]; however, data from other research groups suggest that kinase-dead EGFR is internalized normally like wild type EGFR, but is quickly recycled back to the plasma membrane [96, 97]. Since the mid 1990s, most studies suggest that EGFR kinase activity is required for EGF-induced EGFR internalization. It was reported that EGFR kinase activation is required for the recruitment of EGFR into coated pits [98]. The EGFR activation of c-Src tyrosine kinase has been implicated in the regulation of the clathrin-dependent endocytosis of EGFR through the ability to phosphorylate clathrin heavy chain [99]. The EGFR activation of Eps15 has been shown to be required for the internalization of EGFR [100]. The inhibition of EGFR kinase activity by AG1478 and PD158780 was shown to block EGFR internalization [101, 102].

While most studies indicate that EGFR kinase activity is essential for EGF-induced EGFR internalization [94, 95, 98-102], it was shown recently that EGFR kinase activity is not required for EGF-induced EGFR internalization [103-107]. Inhibition of EGFR kinase activation by the specific inhibitor AG1478 and PD158780 in BT20, MDCK, Cos7 and Hela cells did not block EGFR internalization. When transiently expressed in 293T cells or stably expressed in CHO cells, a kinase-dead EGFR (EGFR K721A), was internalized following EGF stimulation in a similar pattern to wild type EGFR, which indicates that EGFR kinase activation is not required for EGFR internalization [103-107].

If kinase activity is not necessary for EGF-induced EGFR endocytosis, an EGF-induced event before or independent of EGF-induced EGFR kinase activation must be responsible for mediating EGF-induced EGFR endocytosis. The only significant event induced by EGF before the activation of EGFR kinase is the dimerization of EGFR. It is well established that receptor dimerization is critical for EGFR kinase activation [108]. In fact, it is generally believed that the only function of receptor dimerization is to allow the activation of EGFR kinase and the trans-autophosphoylation of the two receptors. However, it was shown recently that EGFR dimerization is necessary and sufficient to stimulate EGFR internalization, independent of EGFR kinase activation [105]. EGF-induced EGFR dimerization in the absence of kinase activation is sufficient to stimulate EGFR internalization. Non-ligand-induced dimerization of EGFR without kinase activation is sufficient to stimulate EGFR internalization. Moreover, the inhibition of EGF-induced EGFR dimerization by deleting the receptor dimerization loop abolishes EGF-induced EGFR internalization [105]. It has also been reported that the crosslinking of two EGFR with antibody stimulates the endocytosis of EGFR without activating EGFR kinase [109]. How dimerization may mediate EGFR endocytosis independent of its role in EGFR kinase activation is not known. Several possibilities have been suggested. It is possible that EGF-induced EGFR dimerization causes necessary conformational changes of the receptor to expose the cryptic internalization codes. Alternatively, the internalization regulating

proteins essential for EGFR internalization may have a dimeric nature and can only bind to dimerized EGFR [105].

Many studies have also focused on the role of EGFR C-terminus in EGFR internalization (Fig. 4). The EGFR mutants truncated from the C-terminus to residue 991 [110] or to residue 973 [111] are internalized inefficiently and the mutant truncated at residue 958 is not internalized [110]. Simultaneous point mutation of the five-tyrosine residues (Y992, Y1068, Y 1086, Y1148 and Y1173) to phenylalanines significantly reduces EGFR internalization [112]. EGFR is co-immunoprecipitated with adaptin AP-2 [88]. The binding between EGFR and AP-2 is mediated by EGFR amino acid residues 970-991, especially Y974 [113, 114]. This interaction accelerates EGFR internalization when EGFR is expressed at high levels, but is not required for EGFR internalization when EGFR is expressed at low levels [83, 113, 114]. A 15-amino acid domain (residues 943-957) was found to be essential for binding sorting nexin-1 (SNX1) which is involved in targeting EGFR to lysosome [115], but not EGFR internalization. It was shown that the EGFR C-terminal sequences from 992 to 1044 are essential for mediating EGF-induced EGFR internalization with or without the inhibition of EGFR kinase activation [105]. It was further shown that EGFR residues 1005-1017, especially the di-leucine ^{1010}LL^{1011} is required for EGF induced rapid internalization of full length EGFR and the role of ^{1010}LL^{1011} in EGFR internalization is independent of EGFR kinase activation [106]. The identification of ^{1010}LL^{1011} as essential for EGFR internalization is very interesting. EGFR di-leucine motif ^{1010}LL^{1011} proceeded with TSRTP, which is different from the two classes of di-leucine-based sorting signals described above. Two di-leucine motifs including ^{679}LL^{680} and ^{1010}LL^{1011} have been implicated in EGFR sorting. It was reported that ^{679}LL^{680} is required for the efficient transport of EGFR to lysosomes and for the retention of EGFR in endosomes [116, 117]. It was also shown that ^{1010}LL^{1011} is critical in the tyrosine phosphorylation of β2 subunit of clathrin adaptor complex AP-2 and is required for EGFR degradation [118]. The only data suggesting a possible role of ^{1010}LL^{1011} in EGFR endocytosis is that it regulates the slow endocytosis of a mutant EGFR truncated at amino acid 1022 [119]. However, other data from the same group showed that ^{1010}LL^{1011} is not involved in the endocytosis of full length EGFR [118, 119].

The role of various EGFR binding proteins in EGFR endocytosis has also been extensively studied. Some proteins that bind to pY sites of EGFR have also been implicated in EGFR endocytosis. These proteins including Grb2, Eps15, PLD, Cbl, Rin1, and Src [41, 99, 100, 119-123]. Grb2 regulates EGFR endocytosis, possibly through its SH3 domain interaction with dynamin [120]. Knocking-down Grb2 with siRNA also blocks EGFR endocytosis [119, 124]. EGF receptor endocytosis is dependent upon PLD and the PLD1 regulators, protein kinase C alpha and RalA [125]. Tyrosine phosphorylation of Eps15 is necessary for the internalization of EGFR [100]. Eps15 functions as a scaffolding adaptor protein and is involved in both secretion and endocytosis. Eps15 has been shown to bind to AP-1 and AP-2 complexes, to inositol lipids, and to several other proteins involved in the regulation of intracellular trafficking [126]. Phosphorylation of clathrin heavy chain by Src facilitates EGFR endocytosis [99]. Rin1 binds to EGFR and regulates EGFR endocytosis through its SH2 domain [123]. Although it is generally agreed that Cbl acts to negatively regulate EGFR

activity by promoting the intracellular trafficking and degradation of EGFR, it is still disputed whether Cbl binding or Cbl-mediated ubiquitination is altogether required for ligand-induced EGFR endocytosis [122, 127]. Some recent data indicate that Cbl-mediated ubiquitin of EGFR is not required for EGFR endocytosis [127, 128]. While PI3K is required for β-PDGFR endocytosis and down-regulation [129-131], PI3K activity is not required for EGFR endocytosis [132].

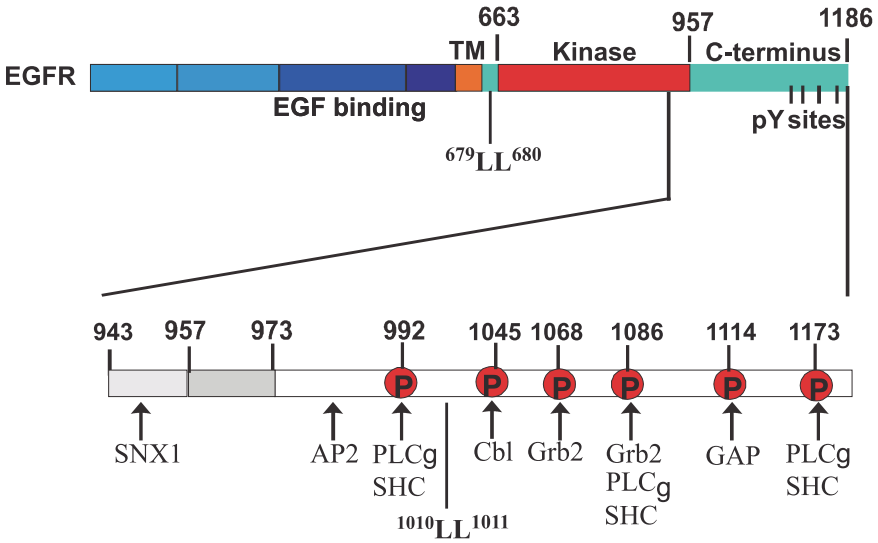

Figure 4. Internalization and sorting signals within EGFR intracellular domain.

Strong evidence suggests that CME is the major pathway of EGFR endocytosis. EGF and EGFR are found concentrated in CCP and CCV. EGFR endocytosed with a rate similar to those of other receptors that are internalized by CME, such as LDL and transferrin (Tfn). Knockdown of clathrin heavy chain or dynamin by RNA interference (RNAi) inhibits EGFR endocytosis [18]. Although CME is certainly an extremely important endocytic mechanism, accounting for a large proportion of endocytic events, an ever expanding array of cargos has been show to undergo endocytosis in clathrin-independent manner [62, 133]. Clathrin-independent endocytosis itself has been further dissected into seemingly distinct pathways, based on the reliance of these pathways on certain proteins and lipids, their differential drug sensitivities and their abilities to internalize particular cargos [134]. Many NCE pathways have been reported including caveolar-type endocytosis, CLIC/GEEC-type endocytosis, the putative flotillin-associated endocytic structures, phagocytosis, macropinocytosis, dorsal ruffles (or waves), and entosis [62, 63]. New evidence suggests that EGF-induced EGFR endocytosis may also be mediated by NCE. NCE of EGFR via dorsal waves was observed in several types of cells [135]. This pathway required the activity of the EGFR kinase, PI3K and dynamin [135]. The NCE of EGFR involving cholesterol-rich lipid rafts and/or caveolar has also been reported [136]. This cholesterol-dependent endocytosis was observed under conditions of high EGFR occupancy by EGF in Hela cells. All of the reported NCE pathways

are significantly slower than CME, although they are faster than constitutive receptor endocytosis [18].

4. Regulation of EGFR signalling by endocytosis

Endocytosis is essential for cells to perceive extracellular signals and transduce them in a temporally and spatially controlled fashion, directly influencing not only the duration and intensity of the signaling output, but also their correct location. It is well established that the endocytosis of EGFR from the plasma membrane to lysosomes results in the degradation of the receptor, which can attenuate receptor signaling and may even be conceived of as a tumor suppressor pathway [19]. On the other hand, accumulated evidence suggests that the internalized EGF-EGFR complex may maintain its ability to generate cell signaling from endosomes [19-23].

4.1. Downregulation of EGFR by endocytosis

Endocytosis has been recognized as the most significant pathway to downregulate EGFR by removing the receptor from the cell surface for degradation in lysosomes [137]. This downregulation of EGFR is a complicated and tightly regulated process. During this process the EGFR-containing internalized vesicles mature into multivesicular bodies (MVBs), which then fuse with lysosomes to allow degradation of their content. This was first shown by Cohen and his colleagues, who observed that ferritin-conjugated EGF was rapidly internalized upon binding to EGFR and trafficked to MVBs within 15 minutes exposure of cells to ligand [87, 138]. The impaired endocytic downregulation of signaling receptors is frequently associated with cancer, since it can lead to increased and uncontrolled receptor signaling [139].

The role of endocytosis in the downregulation of EGFR signalling is best illustrated by the findings that the inhibition of EGFR endocytosis frequently leads to cancer. The best characterized EGFR mutant with impaired endocytosis is EGFRvIII. EGFRvIII has been implicated in many types of tumors [140-145]. EGFRvIII is a mutant EGFR with the deletion of amino acid residues 6–273 in the extracellular domain of EGFR. This results a truncated 145 kDa receptor with a non-functional ligand binding pocket and no dimerization arm. In spite of not binding any ligands, the receptor is constitutively active [146], and is able to activate Ras-Erk1/2 and PI 3-kinase-Akt pathways [142, 147]. In concordance with this, EGFRvIII was shown to transform fibroblasts and to enhance the proliferation and/or tumorigenicity of cells both in vivo and in vitro [142, 148-152]. The constitutive activity may be important for tumorigenicity, but impaired downregulation certainly enhances the effect. Two recent reports show that EGFRvIII is not degraded in cells with endogenous levels of Cbl, instead, internalized EGFRvIII is recycled back to the plasma membrane [153].

It is generally accepted that ErbB2 avoids efficient endocytic downregulation [154-158], which contributes to its important role in the development of various cancers [2, 10]. As ErbB2 and the EGFR-ErbB2 heterodimers are impaired in EGF-induced endocytosis [158], EGFR-mediated cell signaling are significantly sustained in the cells with overexpressed

ErbB2 due to the formation of EGFR-ErbB2 heterodimers. EGFR signaling can also be sustained if the molecular machinery normally involved in receptor downregulation does not function optimally. Indeed, several mutations of such proteins have been found in tumors, including Cbl, TSG101 (an ESCRT-I subunit), and VPS25 (an ESCRT-II subunit) (recently reviewed in [159]. In conclusion, endocytic impairment may be a returning theme of oncogenic EGFR mutants.

The role of endocytosis in the downregulation of EGFR signalling is also frequently and successfully explored as a therapy for cancer. Since the lack of endocytic downregulation is an emerging theme in ErbB cancer biology, it is evident that the stimulation of ErbB endocytosis and lysosomal degradation is an attractive means to inhibit tumor growth. Polyvalent antibodies have been developed to stimulate EGFR and other ErbB endocytosis by crosslinking the receptors together [156, 160]. One good example is trastuzumab (Herceptin). Trastuzumab is a humanized recombinant mAb that binds to the extracellular domain of ErbB2 protein [161, 162]. Currently, it is the only ErbB2-targetted therapy approved by FDA for metastatic breast cancer treatment [163]. Although several recent studies suggest that Trastuzumab does not induce the endocytosis of ErbB2 to a significant degree [154, 156, 157], the dominating opinion has been that Trastuzumab causes endocytic downregulation of ErbB2 [2, 164-166].

Cetuximab is an antibody targeting EGFR that is currently used in treatment of colorectal cancer and head and neck cancer [167]. Several studies have shown that Cetuximab induces the internalization of EGFR [109, 168]. Cetuximab-induced EGFR internalization is independent of receptor tyrosine kinase activity, and it is both slower and less efficient in terms of receptor downregulation than ligand-induced endocytosis [109]. At present, the knowledge of mechanisms underlying antibody-mediated endocytic downregulation is relatively sparse. A useful observation is that extensive antibody-based crosslinking of ErbB receptors is far more efficient at inducing ErbB endocytic downregulation than single antibodies are [156, 160]. Crosslinking can either be done using antibodies that form multivalent aggregates via secondary antibodies or gold particles (Hommelgaard2004}, or by a more clinically relevant approach using combinations of monoclonal antibodies against distinct epitopes in an ErbB receptor [160]. Thus, whereas the administration of Trastuzumab alone did not induce significant ErbB2 endocytosis, the combination of Trastuzumab with another monoclonal antibody to ErbB2 was very efficient at downregulating ErbB2. In addition, the combination of two antibodies was much more efficient at inhibiting tumor growth in a mouse model compared to Trastuzumab administered alone [160, 166].

Although the endocytic downregulation of EGFR has been mostly attributed to clathrin-dependent endocytosis [18], other endocytic pathways have also been proposed during recent years, especially following stimulation with high concentrations of EGF [136]. The concentration of EGF varies greatly throughout the human body. The EGF concentration in most tissue fluid is about 1–2 ng/ml, but it is much higher, up to 100 ng/ml or more, in tubular duct lumens of kidney, salivary glands, and the mammary gland [87, 169]. Normally, EGFR is not reached by the high luminal concentrations of EGF in these systems,

since the receptor is present at the basolateral site of the epithelial cells. However, during wound healing or malignant transformation, the tight junctions disappear and allow the high concentrations of EGF to access the receptor [170]. Very high EGF concentration can also be found in solid tumors [171]. It was reported that at high concentrations of EGF (20 ng/ml) the receptor became ubiquitinated and was to a high degree internalized by caveolae [136]. Incubation of epithelial cells with 30 ng/ml of EGF for 5–20 min resulted in an eight to tenfold increase in the number of plasma membrane caveolae due to EGF-induced tyrosine phosphorylation of caveolin-1 [172]. Moreover, live cell imaging revealed increased dynamics of green fluorescent protein (GFP)-tagged caveolin upon stimulation of cells with 30 ng/ml EGF. Thus, some studies suggest a role of caveolae in EGFR endocytosis. More interesting, it was further revealed by Sigismund et al that EGFRs internalized via CME are not targeted for degradation, but instead are recycled to the cell surface. By contrast, clathrin-independent internalization preferentially commits the receptor to degradation [173]. A prior study has shown that TGFβ receptor is internalized by two distinct endocytic pathways, clathrin-mediated endocytosis leading to TGFβ receptor signaling and lipid-raft-mediated endocytosis leading to the degradation of TGFβ receptor [174].

4.2. Signalling endosomes

The concept of EGFR signalling from endosomes or "signalling endosomes" has been gradually developed. Early evidence to support signalling from endosomes was reported in middle to late 1980s. These researches showed that internalized EGFR is autophosphorylated and catalytically active [175-177]. Various signaling molecules that regulate Ras activity, including Grb2, SHC, Sos and GAP, are co-internalized with EGFR into endosomes and remain associated with the receptor in endosomes [20, 178-181]. Afterwards, more results confirmed the interaction between EGFR and various signaling proteins in endosomes [182-186].

The major evidence supporting endosomal EGFR signalling came from endocytosis inhibition experiments. Since the mid 1990s, researchers have developed many ways to inhibit EGFR endocytosis and then examine the effects on cell signalling. These experiments have yielded mixed results regarding what signalling pathways activated by endosomal EGFR and the physiological relevance of EGFR signaling from endosomes. The inhibition of EGFR endocytosis by a dominant-negative mutant dynamin enhances the activation of PLC-γ1 and cell proliferation, but decreases ERK activation [187]. In a study of EGFR transactivation by G-protein coupled receptors, it was found that the inhibition of EGFR endocytosis by either mutant dynamin or β-arrestin abolished ERK activation [188, 189]. The inhibition of EGFR endocytosis by phospholipase D also blocks EGF stimulated-ERK activation [125]. However, none of these researches provided a mechanism to explain why activated EGFR at the plasma membrane is unable to activate ERK. On the other hand, other research showed that the inhibition of EGFR internalization enhances ERK activation [190, 191]. EGFR efficiently activates mitogen-activated protein kinase in HeLa cells and Hep2 cells, which is conditionally defective in clathrin-dependent endocytosis by overexpressing dominant negative dynamin [191]. Sprouty2 attenuates EGFR ubiquitination and

endocytosis, and consequently enhances Ras/ERK signalling [190]. Initially, in the few cases where biological end points were measured, inhibition of endocytosis did not result in the attenuation of biological effects [187, 192]. These results argue against a physiological relevance of endosome-originated signals [193].

The controversy over endosomal signaling and its physiological relevance is in part due to the limitation of current approaches. For example, while it has made significant contribution and remains a powerful tool to study endosomal signaling, this endocytosis-inhibition approach has its limitations. While the inhibition of EGFR endocytosis eliminates endosomal signaling, the retention of EGFR at the cell surface also enhances signaling from the plasma membrane. Thus, it is difficult to determine whether the observed effects are due to the lack of endosomal signaling or due to prolonged plasma membrane signaling. Blocking EGFR endocytosis by mutant dynamin or β-arrestin affects all endocytic events mediated by these factors. Thus, it is difficult to determine whether the observed effects are due to the inhibition of EGFR endosomal signaling or due to a broad inhibition of endocytosis. Moreover, this approach is not suitable for studying the dynamics of endosomal signaling. None of these approaches offered mean to get activated receptors inside a cell without initial activation at the cell surface [194]

In the early 2000s, a novel system was established to allow the specific activation of endosome-associated EGFR without the initial activation at the plasma membrane and without disrupting the overall endocytosis pathway. To specifically activate endosomal EGFR, cells were treated with EGF in the presence of AG1478, a specific EGFR tyrosine kinase inhibitor, and monensin that blocks the recycling of EGFR. This treatment led to the internalization of inactive EGF-EGFR complex into endosomes. The endosome-associated EGFR was then activated by removing AG1478 and monensin. No surface EGFR phosphorylation was detected [103, 104]. The specific activation of endosome-associated EGFR was also achieved without using monensin[103, 104]. In this system EGFR follows the same endocytic pathway as the control: EGF receptor is first internalized into Rab5-positive endosomes and eventually traffics to lysosomes for degradation. The only difference is that the EGF receptor is not activated during its internalization from the plasma membrane to endosomes and stops at endosomes until being activated. Thus, this system not only allows the generation of specific endosomal signaling of EGFR, but also under a condition very similar to the endosomal signaling of EGFR following its activation at the plasma membrane. By using this system, it was shown that 1) endosomes can serve as a nucleation site for the formation of signaling complexes, 2) endosomal EGFR signaling is sufficient to activate the major signaling pathways leading to cell proliferation and survival, and 3) endosomal EGFR signaling is sufficient to suppress apoptosis induced by serum-withdrawal [103] and to stimulate cell proliferation [195].

In most cases, the endosomal EGFR signaling is the continuation of EGFR signaling at the plasma membrane, serving to maintain EGFR signaling and provide spatial-temporal regulation of EGFR signaling. However, in some cases, specific and novel signaling may be initiated only from endosomes as these signaling events require factors to be brought together by endocytosis. While specific signaling complexes can be assembled through their

recruitment to the early endosomal resident protein RAB5, there are no convincing examples that specific and novel signaling is initiated from endosomes in the context of EGFR signaling. However, it is well illustrated in TGFβ signaling that specific and novel signaling may be initiated only from endosomes. TGFβ receptors (TGFβR) become phosphorylated at Ser residues and are internalized by endocytosis following ligand binding. Once localized into endosomes, TGFβR can bind to SMAD anchor for receptor activation (SARA). The protein complex induced phosphorylation of the transcription factors SMAD1 or SMAD2 by their Ser/Thr kinase receptors. Upon phosphorylation, SMADs are released into the cytoplasm, bind to a cofactor (SMAD4), enter the nucleus, and promote gene transcription [137, 174].

Together, it is clear that EGFR signals from both the plasma membrane and the endosomes, and that the signals from both locations are able to activate major signaling pathways, stimulate cell proliferation, and promote cell survival. However, following EGF stimulation, activated EGF receptors only stay at the plasma membrane briefly (5-10 min), but stay in the endosome much longer (1 h) (Fig. 5) [88]. This argues for a more physiologically important role for endosomal signaling. The plasma membrane EGFR signaling are usually exaggerated by studies with the inhibition EGFR endocytosis, as activated EGFR that stay at the plasma membrane are artificially over extended.

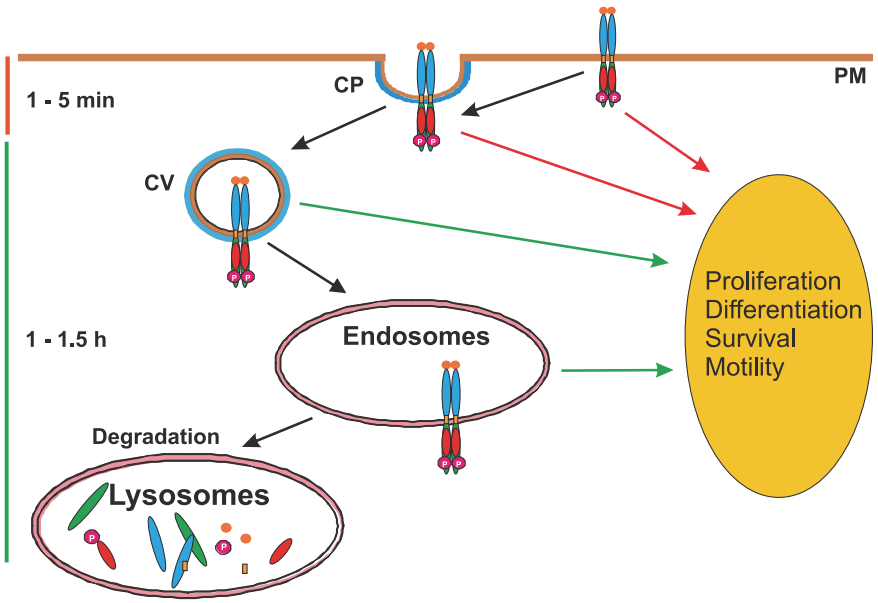

Figure 5. A model to describe the EGFR signaling along its endocytic route. Following its activation at the plasma membrane EGFR continues to signal along its endocytic route till its degradation in lysosome. EGFR signaling from both the plasma membrane and the intracellular endocytic compartment regulates major signaling pathways leading to cell proliferation and survival. The Activated EGFR may stay 1-5 minutes at the plasma membrane and 1 - 1.5 h along the endocytic compartments. PM: plasma membrane. CP: coated pit. CV: coated vesicle.

5. Remaining questions, perspectives and future research

In spite of intensive research and significant progress, many important issues remain unsolved regarding EGFR endocytosis and its regulation of cell signaling. One issue is clathrin-mediated endocytosis vs non-clathrin-mediated endocytosis. Significant evidence supportting the presence of non-clathrin mediated endocytosis and EGFR also internalized through non-clathrin mediated endocytic pathways. However, the mechanisms dictating which endocytic pathways EGFR follows under various conditions are far from clear and the functions of these different endocytic pathways are not clear either. While a few recent researchers showed that EGFR undergoes non-clathrin mediated endocytosis at high EGF concentrations and leads to EGFR degradation [137], the extensive data that support the role of chathrin-mediate endocytosis in EGFR internalization and degradation in lysosomes in the past several decades are mostly obtained at high EGF concentrations. A recent study showed that during cell mitosis, EGFR follows non-clathrin mediated endocytic pathway under both low and high EGF concentrations [107], which suggest that EGF concentration is, at least, not the only factor dictating the entry of EGFR into different endocytic pathways. It is also difficult to explain why cells choose the much slower non-clathrin mediated endocytosis to degrade EGFR, because it provides the heavily phosphorylated EGFR too much time to signal before degradation.

Another issue is the role of EGFR kinase activity in EGFR endocytosis. Both of the opposing claims that EGF-induced EGFR endocytosis is dependent on EGFR kinase activity and that it is independent of EGFR kinase activity are supported by many data. It is difficult to reconcile the differences in the literature. However, a recent piece of research may shed some light. It was recently reported that EGF-induced EGFR endocytosis is independent of EGFR kinase activity during interphase, but is dependent on EGFR kinase activity during mitosis [107]. During mitosis, EGF-induced EGFR endocytosis is slower and independent of clathrin [107]. As previous research never distinguished the cells at interphase from cells at mitosis and at any given time there is a portion of cells at mitosis, the reported results are always a combination of kinase-independent and kinase-dependent endocytosis. Depending on the cell type and experimental conditions, the data may vary significantly. Moreover, EGFR may also undergo both kinase-dependent and kinase independent endocytosis during interphase depending on the cell type and experimental conditions. It has been shown that antibody-induced EGFR endocytosis is independent of EGFR kinase activity [109]. Future research is needed to elucidate the molecular mechanisms underlying kinase-independent and kinase-dependent endocytic pathways.

Last, but not least, the function and significance of EGFR signaling from endosome vs EGFR signaling from the plasma membrane provides room for further research. It is clear that EGFR signals from both the plasma membrane and the endosomes, and that the signals from both locations are able to activate major signaling pathways, stimulate cell proliferation, and promote cell survival. However, the extent of the difference between these two signals is unclear. So far, the results have come from either the comparison between endosomal signaling and standard EGFR signaling, or the comparison between plasma membrane signaling and standard EGFR signaling. A direct comparison between

endosomal EGFR signaling and the plasma membrane EGFR signaling is needed to define the functional difference and their physiological significance of these two signals. The spatio-temporal dynamics of EGFR signaling in controlling cell function has become a new focus of current research. EGFR signaling along the endocytic route from the plasma membrane to endosomes allows a vigorous regulation of spatio-temporal dynamics of EGFR signaling.

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