Minireview

Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases

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Abstract **The major process that regulates the amplitude and kinetics of signal transduction by tyrosine kinase receptors is endocytic removal of active ligand–receptor complexes from the cell surface, and their subsequent sorting to degradation or to recycling. Using the ErbB family of receptor tyrosine kinases we exemplify the diversity of the down regulation process, and concentrate on two sorting steps whose molecular details are emerging. These are the Eps15-mediated sorting to clathrin-coated regions of the plasma membrane and the c-Cbl-mediated targeting of receptors to lysosomal degradation. Like in yeast cells, sorting involves not only protein phosphorylation but also conjugation of ubiquitin molecules. The involvement of other molecules is reviewed and recent observations that challenge the negative regulatory role of endocytosis are described. Finally, we discuss the relevance of receptor down regulation to cancer therapy. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Clathrin; Growth factor; Oncogene; Review; Signal transduction; Tyrosine kinase; Ubiquitin

1. Introduction

Exposure of cells or tissues to a variety of hormones and growth factors almost invariably leads to disappearance of specific binding sites from the cell surface. This phenomenon, which is due to accelerated endocytosis of ligand–receptor complexes, was termed 'down regulation'. Down regulation of one group of receptor tyrosine kinases (RTKs), those that bind growth factors sharing an epidermal growth factor- (EGF-) like motif, is one of the most extensively analyzed. The four members of this group, ErbB-1 through ErbB-4, are transmembrane proteins whose large extracellular domains bind specific growth factors, whereas their intracellular domains are endowed with tyrosine kinase activity (reviewed in [1]). The monomeric forms of ErbB proteins are catalytically much less active than the ligand-induced dimers, whose autophosphorylation recruits diverse phosphotyrosine binding proteins to initiate signal transduction. Because the four ErbB proteins can form both homo- and heterodimers, and each receptor can recruit a specific set of signalling proteins, this configuration allows enormous potential for signal diversifica-tion. Moreover, one ErbB protein, namely ErbB-3 is devoid of enzymatic activity [2], whereas ErbB-2 seems to function sole-ly as a low affinity co-receptor [3]. Thus, signalling by ErbB receptors and their many ligands may be considered in terms of a layered network whose output depends on combinatorial interactions.

Uniquely, the ErbB network can be tracked in evolution to a primordial simple module in worms. The single ErbB ortho-log of *Caenorhabditis elegans* is activated by only one ligand, called Lin3, and it transmits signals primarily through the Ras pathway. This linear pathway evolved throughout evolution to form a richly interconnected network, whose complexity derives from the existence of many ligands and four receptors capable of forming 10 dimeric combinations. Although signal-ling down-stream of all four mammalian ErbB proteins is funneled into the Ras pathway, variation exists in terms of the specific repertoires of phosphotyrosine binding proteins that are recruited to each receptor. Moreover, the various dimeric receptors differ in the potency of mitogenic signals, presumably because each ErbB protein follows a unique path-way of endocytosis and down regulation. For example, ErbB-1 is rapidly internalized and degraded following activation by some ligands, but internalization of ErbB-2 and the two neu-regulin receptors, ErbB-3 and ErbB-4, is relatively slow [4,5]. Because ErbB-3 is devoid of enzymatic activity and this func-tion is essential for degradation of internalized receptors [6], this neuregulin receptor recycles back to the plasma mem-brane, perhaps after unloading its ligand in an endosomal compartment [7].

A wealth of experimental evidence has established the no-tion that the kinetics of signalling by ligand-activated recep-tors determines not only the amplitude of the output but also its specificity (e.g., mitogenesis or differentiation) [8]. Consis-tent with this paradigm, a mutant ErbB-1 whose endocytosis is impaired can deliver oncogenic signals [9], and several on-cogenic animal viruses impair endocytic removal of active ErbBs from the cell surface. Examples include the E5 protein of human papilloma virus, which blocks an endosomal ATP-ase, thus shunting internalized receptors to the recycling path-way [10]. Poxviruses encode multiple EGF-like ligands that bind with relatively low affinity to ErbB proteins. However, because the viral ligands cause only limited receptor down regulation, their mitogenic potency is enhanced relative to the mammalian counterpart [11]. Retroviruses present a vari-ety of mechanisms that help them evade receptor down regu-lation: Oncogenic Ras proteins appear to slow down the rate

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of internalization, whereas the oncogenic v-ErbB receptor encoded by the avian erythroblastosis virus is devoid of a phosphorylation site essential for targeting to lysosomal degradation [12].

Despite their importance, negative regulatory processes are less understood than the steps involved in signal generation and propagation to the nucleus. Lessons derived from the endocytic pathways followed by cargo receptors like the transferrin receptor (TfR) and the low density lipoprotein (LDL) receptor are only partially relevant to signalling receptors like ErbBs. Nevertheless, the list of proteins that participate in receptor inactivation is steadily increasing and we now begin to understand their interactions along the endocytic routes. After dealing with endocytosis in simple eukaryotes, namely yeast cells, we concentrate on two sorting processes that determine receptor down regulation. These are sorting of receptors to internalization through the clathrin-coated pit and their later interaction with a machinery that determines lysosomal degradation.

2. Lessons from yeast

Genes that are not essential for viability can be deleted from the yeast genome and thus enable direct examination of their cellular role. The usefulness of this approach has been repeatedly exemplified by the isolation of mutant yeast cells defective in certain steps of endocytosis. Recent ultrastructural and biochemical approaches suggest that the general organization of endocytic traffic inside yeast cells resembles that of mammalian cells. Both early and late endosomes have been morphologically and biochemically identified in yeast [13,14], and the molecular machinery required for vesicular transport in yeast does not seem to be fundamentally different from the mammalian machinery [15].

Due to the lack of refined assays to investigate intracellular post-endocytic steps, this phase is less understood than the internalization step, which has been thoroughly investigated in yeast. Interestingly, most yeast transmembrane proteins, even transporters specific for certain metabolites [15,16], seem to undergo constitutive endocytosis. However, some of these endocytic systems display a large increase in the rate of endocytosis upon varying growth conditions or, in the case of pheromone receptors, by the addition of a ligand to the growth medium. Several genes that are potentially essential for accelerated endocytosis were identified. Consequently, some striking similarities and differences between yeast and mammalian internalization became apparent. One such protein is the clathrin heavy chain (CHC), a main component of the clathrin coat and a major player in membrane sorting. Surprisingly, inactivation of the gene (CHC1), using a temperature sensitive allele, resulted in moderate (50%) reduction in the rate of a-factor internalization [17]. This partial effect of a clathrin mutation on endocytosis in yeast may be explained by the existence of two parallel internalization pathways: a clathrin-dependent route as well as a clathrin-independent pathway, as is the case in mammalian cells.

Another aspect relates to the classical endocytic signals identified in mammalian plasma membrane proteins (e.g. YXXΦ, where Φ is a bulky hydrophobic amino acid, MPXY, and a di-leucine motif). These motifs bind specific subunits of the AP-2 adapter complex of clathrin, but only rarely have similar signals been identified in yeast. One example is the linear sequence NPFTD, which is required for ligand- and clathrin-dependent endocytosis of the a-factor receptor [18]. Similarly, the di-leucine motif is required for endocytosis of the Gap1p permease [19]. In neither case have the interacting molecules been identified. However, in several other cases the endocytic signals identified in yeast correspond to motifs required for protein ubiquitination (reviewed in [20]). The identified ubiquitination signals are relatively long acidic sequences. At least in the case of Ste2p, Ste3p and Fur4p, the acidic nature seems to be modulated by phosphorylation [21–23]. A phosphorylation-based recognition signal for endocytosis is an appealing possibility in both yeast and mammals, because ubiquitination and/or endocytosis of many receptors and transporters are often regulated by ligands, substrates or extracellular nutrients.

Similar to clathrin, the involvement of dynamin in endocytosis has been extensively documented in animal cells, but its role in yeast is rather limited. Although three dynamin homologs were identified in yeast, Mgm1p, Vps1p and Dnm1p, none is absolutely required for internalization of membrane proteins [24,25]. By contrast, Pan1p, the yeast homolog of the mammalian EGF-receptor protein substrate Eps15, is essential for normal endocytosis. The polyvalent structure of Pan1p implicates it as a key regulator that may transiently recruit components of the endocytic machinery [26]. An interesting genetic interaction between Pan1p and the ubiquitin ligase Rsp5p (the yeast homolog of human Nedd4) has been identified [27]. This WW and HECT domain protein is required for endocytosis of many yeast proteins [27–29], and although its biochemical interaction with Pan1p is still uncharacterized, lessons accumulating with Nedd4 raise the possibility that Pan1p acts as an adapter connecting the ligase to potential ubiquitination substrates. That Pan1p may itself be ubiquitinated by Rsp5p is suggested by the observation that Eps15 is ubiquitinated in mammalian cells [30].

3. Major endocytic routes of membrane receptors

Several morphologically and functionally different types of endocytic pathways exist in animal cells. Phagocytosis represents the uptake of solid particles (>0.5 μ diameter) that must bind to specific plasma membrane receptors capable of triggering their own endocytosis, usually by causing the formation of F-actin-driven pseudopods that envelop the bound particle. On the other hand, pinocytosis enables uptake of extracellular fluid, macromolecules and solutes bound specifically or non-specifically to the plasma membrane. A third pathway, receptor-mediated endocytosis, is the selective adsorptive uptake of specific macromolecules bound to plasma membrane receptors (see Fig. 1). This process involves constitutive formation of small (<0.2 μ diameter) vesicles, which is usually preceded by the formation of clathrin-coated vesicles (CCVs). The basic components of the clathrin coat have long been known [31,32]. A tri-skelion of three CHCs tightly associated with three clathrin light chains constitutes the assembly unit of the polygonal lattice. A hetero-tetramer (AP-2) consisting of two large (α and β) and two small subunits (σ2 and μ2) mediates assembly of clathrin cages on the plasma membrane. The β subunit interacts with clathrin [33], whereas the μ2 subunit is capable of binding the tyrosine-based endocytic signals that mediate internalization of a number of membrane proteins [34,35].
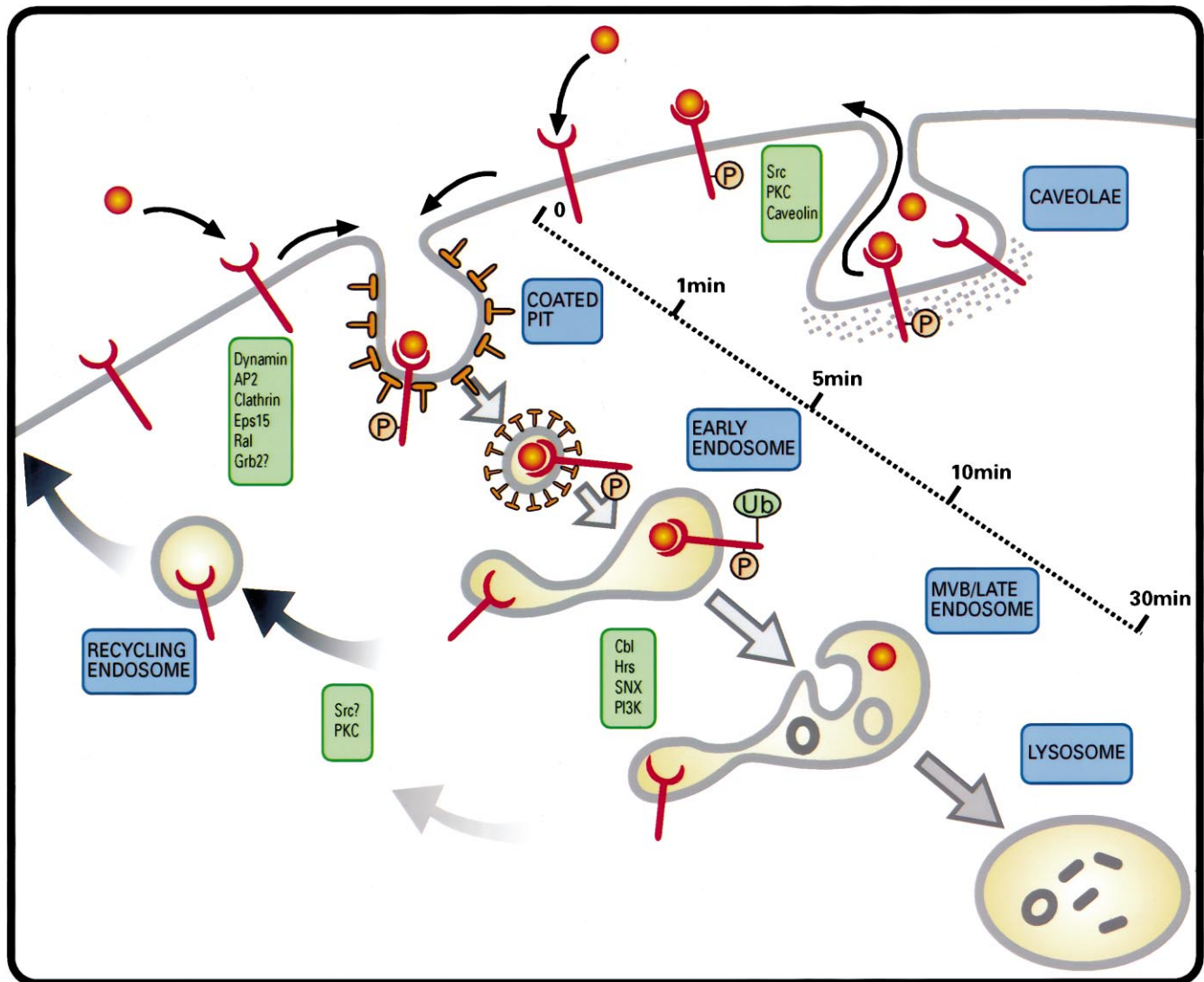
Fig. 1. Model of endocytosis of RTKs based on the trafficking of ErbB-1. The major route of endocytosis from the clathrin-coated pit are marked and the time scale indicated. Unoccupied receptors may be concentrated in caveolae and in non-caveolar regions of the cell surface, but upon ligand binding and activation of the intrinsic kinase, caveolar receptors translocate out of the caveolae. The potential major players of caveolar retention and exit are indicated. The mechanism that allows recruitment of the AP2 complex and a clathrin coat are regulated by a large number of adapters and enzymes, including dynamin and Eps15. Following formation of a clathrin-coated vesicle and its uncoating to form the early endosome, the second major sorting event takes place. This involves sequestration of active receptors in inner vesicular structure of the multi vesicular body. On the other hand, receptors whose ligands are released due to the acidic pH of the endosomal compartment, are directed to tubular structures. The sorting process appears to involve recruitment of c-Cbl, receptor ubiquitination and inward vesicular transport regulated by lipid kinases. Recycling of receptors back to the plasma membrane occurs from most endosomal compartments, but its efficiency is gradually reduced, as the compartment becomes deeper in the cytoplasm. Recycling from the tubular portion of the MVB may involve specific protein kinases such as PKC and Src.

An alternative to clathrin-mediated endocytosis involves specialized forms of rafts, glycosphingolipid and cholesterol-enriched microdomains, termed caveolae. These are small invaginations that exist on the surface of many cell types. The flask-shaped caveolar pit is characteristically 50–80 nm in diameter, highly uniform, and enriched in caveolins, sphingolipids and cholesterol [36–38]. Caveolae are coated with a spiral-shaped striated coat, which is structurally different from clathrin lattices. Molecules internalized through caveolae (reviewed in [38]) may travel to the cytoplasm or to the endoplasmic reticulum. Alternatively, they may be directed to a caveolae derived tubular/vesicular compartment. Caveolae and clathrin-coated pits are specialized to internalize different types of molecules. Therefore, caveolae-mediated, and clathrin-mediated endocytosis are parallel, but non-overlapping, endocytic pathways. Moreover, although several proteins implicated in vesicle trafficking have been localized to caveolar fractions, it is unclear whether tubular or vesicular caveolae ever fuse with endosomes originating in coated pits [39].

4. Receptor sorting to the clathrin-coated vesicle

4.1. Exit from caveolae

In quiescent fibroblasts a relatively large fraction of ErbB-1 is concentrated in caveolae [40–42], but other reports suggested that most of the receptor of overexpressing cells is

confined to the low-buoyant density fraction, representing non-caveolar membrane domains [43]. Structural analysis revealed that the information required for delivery of ErbB-1 to caveolae is contained within the transmembrane and juxtamembrane domains of the receptor, distinct from a caveolin-1 binding domain [44]. In response to EGF, the total number of surface receptors decreases, along with a decline in the percentage of EGF receptors in the caveolar fraction. Depending on the cell type, it takes 3–30 min for ErbB-1 to leave caveolae [41]. The rapid exit appears to require autophosphorylation of at least one of the five major tyrosine residues in the regulatory domain of the receptor, as well as an intact kinase activity [45]. In addition to ligand binding, Src family kinases may control receptor traffic out of caveolae, as a synthetic Src inhibitor can inhibit receptor exit. Consistent with this model, overexpression of Src stimulates an increase in the rate of receptor endocytosis [46]. On the other hand, movement out of caveolae is inhibited by activators of protein kinase C (PKC). Interestingly, overexpression and truncation of ErbB-1 cause a marked phosphorylation of caveolin-1, a major component of caveolae whose exact function is still unclear [47,48]. Noteworthy is the fact that migration from caveolae is uncoupled to internalization through clathrin-coated pits, as blocking clathrin mediated endocytosis does not affect ligand-stimulated depletion of the receptor from the caveolar fraction.

4.2. Interactions with the AP2 recruiting complex

Natural mutations of the LDL receptor helped uncover the determinants needed for recruitment of constitutively internalizing receptors to the clathrin/AP-2 complex [49], and also involved a particular tyrosine residue [50]. Since then, a large variety of internalization signals have been identified by site-directed mutagenesis of various cell-surface proteins. Although structurally heterogeneous, these signals may be divided into two groups (reviewed in [35,51]). The first group is characterized by an essential tyrosine, which is part of the motif NPXY or YXXΦ. Resolution of the crystal structure of an internalization signal (a YXXΦ peptide) bound to the μ2 subunit of AP2 showed that the peptide assumed an extended conformation, and specificity was conferred by hydrophobic pockets that bound the tyrosine and the hydrophobic residues of the peptide [52]. A second group of internalization motifs typically contains a di-leucine sequence, but in some cases one of the leucines may be replaced by an isoleucine, valine or an alanine. In the case of ErbB-1, a stoichiometric complex with AP2 has been attributed to an internalization signal flanked by tyrosine residue 974 [53]. However, mutant receptors lacking the putative AP2 binding site can undergo internalization via clathrin-coated pits [54]. Likewise, the two di-leucine motifs of ErbB-1 may not play a role in internalization of a full-length receptor [55–58]. Presumably, internalization signals allow only low affinity interactions between ErbB proteins and AP2. This may be sufficient for slow constitutive internalization of unoccupied or kinase-defective receptors, but additional interactions may be involved in ligand-stimulated recruitment into coated pits. In line with this scenario, the rapid endocytic pathway of ErbB-1 is saturable [59], but saturation of the endocytic pathway for TFRs does not affect endocytosis of ErbB-1 [60].

4.3. Proteins involved in ligand-regulated recruitment

The three major components of the coated pit are clathrin, AP2 and dynamin. AP-2 drives clathrin assembly and recruits the cytoplasmic tails of constitutively internalizing receptors, as well as ligand-induced receptors. On the other hand, clathrin defines the structure of the pit and dynamin is responsible for fission of the vesicle from the plasma membrane. This large GTPase is thought to act as a mechano-enzyme that mediates the constriction (liberation) of nascent clathrin-coated pits from the plasma membrane during endocytosis [61]. In vitro studies indicate that dynamin binds to membrane-embedded phosphoinositides via its PH domain, and its GTPase activity constructs and fragments membrane tubules capped by clathrin-coated buds [62] (see Fig. 2). A mutant dynamin defective in the GTPase activity blocks ligand-induced endocytosis of ErbB-1, but constitutive internalization is not affected [63]. Unlike constitutively internalizing receptors, which directly interact with AP2, the endocytic signals of ligand-induced receptors may be exposed only upon receptor autophosphorylation. Indeed, kinase activity and a phosphorylation substrate are required for efficient recruitment of ErbB-1 but not TfR [64]. The identity of the substrate(s) remains unknown. However, recent work by Di-Fiore and colleagues implicated an AP2 binding protein, Eps15, in accelerated endocytosis of ErbB-1 [65], and analyses of signalling downstream to the Ral small GTPase attributed a role in endocytosis to another AP2 binding partner, namely RalBP1 [66,67].

4.3.1. Eps15 and Eps15R. These related proteins are endowed with multiple binding specificities: three copies of the EH domain bind to NPF motifs of NUMB and other proteins, while the centrally located coiled coil region allows homodimerization or heterodimerization with other coiled coil proteins such as intersectin. Finally, the COOH-terminal region, which is characterized by repeated DPF tripeptides binds the α subunit of the AP2 complex (reviewed in [68]). Upon activation of ErbB-1, Eps15 is recruited to the plasma membrane [69] and localizes to coated pits [70]. By electron microscopy, Eps15 was found to localize to the rim of the budding-coated vesicle and not to deeper invaginations. The rim is the growing part of the forming pit. During coat assembly the rim is the only site where Eps15 remains associated with AP-2, but once clathrin polymerization has taken place, Eps15 may be excluded from clathrin/AP2 complexes [71]. Consistent with an essential role in receptor-mediated endocytosis, expression of dominant negative mutants of Eps15 or microinjection of neutralizing antibodies inhibited endocytosis of both ErbB-1 and TfR [72,73]. However, tyrosine phosphorylation of Eps15 is required exclusively in the process of ligand-induced receptor internalization. Thus, an Eps15 mutant defective in the major tyrosine phosphorylation site (tyrosine 850) specifically inhibited internalization of ErbB-1, but did not affect internalization of TfR [65]. Because the corresponding phosphopeptide can block internalization, it is conceivable that following ligand binding and elevated phosphorylation of tyrosine 850, an unknown phosphotyrosine binding protein binds to the modified tyrosine and selectively accelerates recruitment of occupied receptors to the AP2 complex. In conclusion, Eps15 may fulfil a dual role; while it is essential for endocytosis of constitutively internalizing receptors, its phos-
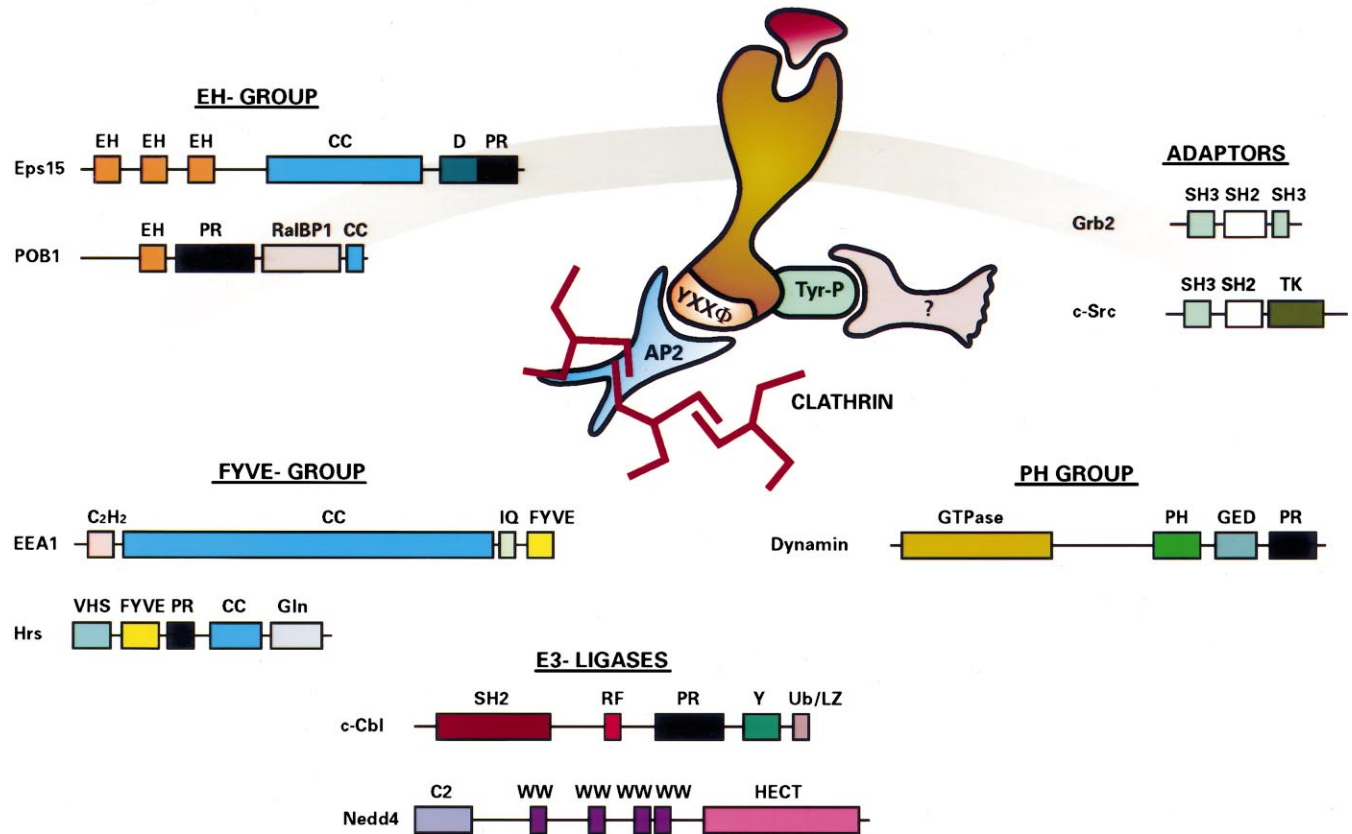
Fig. 2. Molecules involved in sorting RTKs along the endocytic pathway. The transmembrane receptor is schematically depicted in a complex with AP2 and a clathrin cage. The interaction of AP2 with the cytoplasmic part of the receptor is mediated by an endocytic signal such as the YXXΦ motif, but other adapters, whose engagement depends on tyrosine phosphorylation, may enhance recruitment of the AP2 complex. Molecules involved in this sorting event, as well as the sorting that occurs in multi vesicular bodies are grouped and schematically presented. The structural domains are shown in different colors and their names indicated. The abbreviations used are: CC, coiled coil; C2, a phospholipid and calcium binding region; D, a region containing DPF repeats; EH, Eps15 homology; FYVE, a PI3P binding; RF, a RING finger domain; PR, a proline-rich domain; SH, Src homology domain; TK, tyrosine kinase.

phorylation is required only for the rapid, ligand-induced endocytosis of ErbB-1.

4.3.2. RalBP1 and POB1. Ral is a member of the small GTP binding protein family [74,75]. The only known effector protein of Ral, RalBP1, and its own partner POB1, are both implicated in EGF signalling downstream of Ras [76]. Exposure of cells to EGF or to insulin increases the GTP-bound active form of Ral through activation of Ras and its effector, a guanine nucleotide exchange factor for Ral (RalGEF) [77–79]. Active Ral binds to the C-terminal part of RalBP1, a putative GTPase of Rac1 and CDC42 [75]. While the relevance of this GAP activity to endocytosis remains unknown, RalBP1 can effectively recruit the AP2 complex, either directly or through POB1 and Eps15 (see below). The μ2 chain of AP2, but not other coat proteins, binds to the N-terminus of RalBP1, and inhibition of these constitutive interactions blocks endocytosis of both ErbB-1 and TfR [67]. On the other hand, phosphorylation of POB1, a binding partner of RalBP1, Eps15 and epsin, another EH domain protein that participates in clathrin-mediated endocytosis [80], is elevated by EGF. Thus, recruitment of POB1 to the AP2 complex may be involved in the ligand-induced pathway. Indeed, deletion

mutants of POB1 can inhibit endocytosis of both ErbB-1 and the insulin receptor [66]. Presumably, RalBP1 is translocated to the plasma membrane upon stimulation with EGF and subsequent activation of Ras and Ral. Once associated with the plasma membrane, RalBP1 can bind AP2 in a complex manner that involves not only constitutive and ligand-induced interactions, but also the intrinsic GTPase activity.

4.3.3. c-Src and Grb2. A role for c-Src in EGFR-mediated responses has been demonstrated by a number of studies showing that it is required for EGF-induced mitogenesis and tumorigenesis [81,82]. The mechanisms by which c-Src influences the biological action of ErbB-1 are diverse: c-Src may potentiate receptor activity by binding to the receptor and inducing its phosphorylation, resulting in enhanced downstream signalling [81]. Alternatively, there may be a mutual catalytic regulation of the receptor and c-Src [83]. However, accumulating results attribute to Src a role in the endocytic trafficking of RTKs. A significant fraction of c-Src in fibroblasts has been found associated with endosomes [84], and the SH3 domain of c-Src is capable of binding and activating dynamin [85]. Overexpression of Src leads to an increase in the rate of endocytosis of ErbB-1 [46]. The under-

lying mechanism was investigated in cells lacking endogenous Src family members, or in cells treated with the Src inhibitor PP1. These experiments showed that endocytosis of the activated receptor is delayed when Src activity is inhibited [86]. Furthermore, Src activation leads to tyrosine phosphorylation of the CHC at tyrosine 1477, located at a region involved in clathrin assembly. Consequently, clathrin undergoes redistribution to the cell periphery, which may explain how Src is involved in induced endocytosis [86]. The exact involvement of another target of RTKs, Grb2, is less understood. However, this SH3-SH2-SH3 adapter protein (see Fig. 2) binds to a large variety of cellular proteins, including some effectors of endocytosis such as POB1, amphiphysin and synaptojanin. In addition, the SH3 domains of Grb2 interact with dynamin to activate its GTPase in synergy with phosphoinositides [87]. That some of these interactions are necessary for endocytosis of ErbB-1 is indicated by the inhibitory effect of a microinjected fusion protein containing the SH2 domain of Grb2, or the corresponding phosphopeptide ligand [88].

5. Sorting in the multivesicular body (MVB)

Once sorted to clathrin-coated vesicles, internalized receptors are delivered within 2–5 min to a tubular–vesicular network located at the cell periphery (Fig. 1). After 10–15 min, ligand–receptor complexes accumulate in relatively large perinuclear vesicles that contain internal vesicles (MVBs) [89]. These intermediate endosomes are characterized by an accumulation of hydrolytic enzymes, and low internal pH, sufficiently acidic to dissociate some ligands. Studies performed with recycling receptors and kinase-defective mutants of ErbB-1 imply that the MVB is the major site of sorting to lysosomal degradation. Unlike TFRs and kinase-dead ErbB-1 molecules, which are confined to the vesicular portion of MVBs, internalized ErbB-1 molecules accumulate in the inner vesicles of the MVB [6,90]. It is thought that translocation of active ErbB-1 molecules from the perimeter of the MVB to internal vesicles requires phosphorylation of an endosomal substrate that allows, perhaps together with ancillary proteins, removal of the receptor from the recycling pathway. The mechanisms underlying regulation of this critical sorting event currently begin to surface with the identification of candidate molecular players we discuss below.

5.1. c-Cbl

Members of the Cbl family of adapter protein are early prominent substrates for tyrosine phosphorylation by activated receptors for growth factors, cytokines, and immunoglobulins (reviewed in [91]). A single Cbl ortholog, Sli1, exists in nematodes and genetic evidence attributed to it a major negative regulatory role downstream of Let23, the ErbB ortholog of worms [92]. c-Cbl consists of an N-terminal unique Src homology domain (SH2), which mediates binding to tyrosine-phosphorylated receptors [93], and a C-terminal half that carries a long proline-rich domain and several tyrosine and serine phosphorylation sites, serving in constitutive and inducible interactions. A centrally located ring-finger (RF) domain separates the two adapter domains. The RF is missing or defective in two oncogenic forms of c-Cbl, v-Cbl and 70Z-Cbl, suggesting a role in the negative function of c-Cbl. Interestingly, c-Cbl cannot interact with ErbB-3 and ErbB-4, two receptors whose ligand-induced down regulation is im-

paired [94]. Indeed, overexpression of c-Cbl enhances down regulation of ErbB-1 and also increases ligand-induced ubiquitination of this receptor [95]. Recently, c-Cbl was identified as a ubiquitin ligase whose RF recruits an ubiquitin-loaded E2 enzyme [12,96], thus establishing its direct role in ubiquitination of ErbB-1. However, the exact site of action of c-Cbl is a matter of controversy. Evidence derived from experiments with yeast (see above), the growth hormone receptor [97] and blocking ErbB-1 internalization with a dynamin mutant [98] suggested that ubiquitination may be associated with sorting at the plasma membrane. In addition, translocation of c-Cbl to the plasma membrane was observed in macrophages [99]. On the other hand, several groups reported on the endosomal localization of c-Cbl and its co-localization with internalized receptors [95,100,101]. Moreover, the phosphorylation site of ErbB-1 that allows c-Cbl recruitment and down regulation (tyrosine 1045) has been previously mapped by mutagenesis to a lysosomal targeting motif [102]. In support with endosomal sorting, Cbl proteins defective in ubiquitination enhance recycling of ErbB-1 molecules, probably by inhibiting c-Cbl's action [103].

5.2. PI3K

Phosphoinositide 3-kinases phosphorylate inositol lipids at the 3′ position of the inositol ring to generate the 3-phosphoinositides PI(3)P, PI(3,4)P2 and PI(3,4,5)P3. Attempts to clarify the nature of PI3K involvement in membrane traffic in mammalian cells have been largely based on the use of inhibitors of the catalytic activity of PI3K, such as wortmannin. A post-endocytic function has been attributed to PI3K in the case of the receptor for PDGF. Inhibition of PI3K by wortmannin or mutagenesis of the PI3K docking site of the PDGF receptor resulted in altered endocytosis [104]. In both cases internalized receptors remained confined to peripheral endosomal vesicles and escaped translocation to perinuclear endosomes. How exactly PI3K drives vesicular traffic is still unknown, but a recent study revealed necessity of the kinase for structural integrity of the MVB ([105]. Unlike ErbB-3 and ErbB-4, which directly interact with PI3K, ErbB-1 seems to recruit this enzyme only indirectly, either via c-Cbl [106,107], or through ErbB-3 in a heterodimeric receptor complex [108].

5.3. Hrs

Recent research has shown that one way by which lipid kinases affect vesicular transport is by interacting with 3-phosphoinositide binding modules in a broad variety of proteins. Specifically, certain FYVE domains bind PI(3)P, whereas certain pleckstrin homology (PH) domains bind PI(3,4)P2 and PI(3,4,5)P3. One mammalian FYVE-finger protein implicated in trafficking is Hrs, a hepatocyte growth factor-regulated tyrosine kinase substrate [109], which was found to be tyrosine-phosphorylated also in an EGF-dependent manner. The localization of Hrs to an endosomal compartment seems to depend on FYVE-PI3P interactions that may cooperate with a second domain of Hrs [110,111]. Hrs is likely to be a mammalian homolog of the yeast sorter Vps27p, which is essential for vacuolar and endocytic trafficking through a pre-vacuolar compartment [112]. Accordingly, mouse cells that lack Hrs contain abnormally large early endosomes [113], and Hrs over-expression leads to the appearance of large structures containing endosomal markers [110]. These lines of evidence indicate that Hrs specifically influences the dynamics of multi-

ple endocytic compartments, which merge when the protein is overexpressed, perhaps due to promotion of vesicle aggregation or of vesicle fusion [110].

5.4. SNX-1

A yeast two-hybrid system using the core tyrosine kinase domain of ErbB-1 has identified SNX1 and implicated the protein in sorting of the receptor to lysosomal degradation [114]. SNX-1 specifically interacts with a previously identified lysosomal targeting motif, distinct from the c-Cbl's interaction site [115]. The putative sorting molecule contains a region of homology to a yeast vacuolar sorting protein, and overexpression of SNX-1 decreases the amount of ErbB-1 on the cell surface as a result of enhanced rates of constitutive and ligand-induced degradation. Recent studies revealed the existence of a large family of SNX-like molecules that are conserved in yeast and nematodes, and partly associate with the plasma membrane [116].

5.5. PLCγ

To date there is only indirect evidence for the involvement of PLCγ in ErbB-1 trafficking. Immortalized fibroblasts genetically deficient in PLCγ do not show significant effects on ErbB-1 endocytosis [117]. However, a single tyrosine, which serves as a PLCγ docking site on the receptor for the fibroblast growth factor, was found to be important for cellular trafficking [118]. Another clue for a role in endocytosis came from the observation that the SH3 domain of PLCγ is able to bind dynamin in a growth factor inducible manner [119,120].

5.6. PKC

Trans-modulation of ErbB-1 by an active PKC has been attributed to phosphorylation of a single threonine residue at the juxtamembrane domain of the receptor [121]. The modified receptor displays altered kinase activity and ligand binding affinity, and its ligand-induced down regulation is compromised [122]. PKC also affects the unoccupied receptor through enhanced internalization, which is followed by recycling back to the cell surface [123,124]. By using c-Cbl-induced ubiquitination as a marker for transfer from early to late endosomes, it has been recently shown that PKC can inhibit this process, as well as receptor down regulation and degradation [125]. Apparently, PKC-induced phosphorylation at threonine 654 is sufficient to direct incoming receptors to the recycling endosome, whereas phosphorylation at tyrosine residues, through the recruitment of c-Cbl, directs them to the MVB/late endosome. Currently it is not known how PKC activity accelerates internalization and inhibits sorting to lysosomal degradation.

6. Relationships between signalling and receptor trafficking

Ligand-induced receptor internalization has long been considered an attenuation mechanism for signal transduction. However, mounting evidence suggests more complex relationships as receptors internalized in endosomes, or immobilized at submembranal domains are capable of signalling in a surprisingly selective manner.

6.1. Signalling from caveolae

In addition to serving as a gate for entry into the cell, caveolae are the sites where multiple signalling pathways converge. Immunocytochemical, co-immunoprecipitation and cell-fractionation techniques have shown that a number of signalling proteins, including RTKs, such as the receptors for EGF and PDGF, as well as non-receptor kinases and G-proteins, are found associated with caveolae. This suggested that caveolae compartmentalize enzymatic reactions essential for signalling from the plasma membrane (reviewed in [38,126]). The hypothesis that caveolae play a crucial role in signal transduction by pre-assembling inactive signalling complexes ready for rapid activation in response to extracellular signals, is based on the following findings. Caveolin-1 can interact with the catalytic domain of many resident proteins of the caveolar fraction through the caveolin scaffold domain. These include not only ErbB-1 [44] but also c-Src and the Gα subunit of heterotrimeric G-proteins. In fact, isolated caveolae contain all essential components required for MAP kinase activation [127], and in intact cells, both PDGF [42] and EGF [41] stimulate the recruitment to caveolae of multiple signal transducing molecules, as well as the migration of the respective receptor out of caveolae [45]. Depletion of cholesterol, a major building block of caveolae, causes hyper-activation of the MAP kinase [128]. By itself, caveolin-1 is a cholesterol binding protein [129], which has a key role in controlling the level of cholesterol at the plasma membrane [130]. A dominant negative mutant of caveolin-1 is unable to mediate cholesterol trafficking to the plasma membrane, and it can block the action of H-Ras, but not K-Ras [131]. Lastly, caveolin is down-regulated and caveolae are reduced in numbers in transformed fibroblasts [132]. In accordance, conditional expression of caveolin-1 can abrogate the transformed phenotype [133], and antisense depletion of caveolin-1 in intact cells results in cell-transformation [134].

6.2. Signalling from endosomes

Evidence for the existence of highly tyrosine-phosphorylated ErbB-1 molecules in endosomes came from fractionation of rat liver [135]. This state has also been visualized directly by immunoelectron microscopy of A431 cells overexpressing the receptor [136]. The cytosolic orientation of the tyrosine-phosphorylated tail and the presence of an active receptor in endosomes for a prolonged period of time suggests that the receptor may continue to signal after internalization. Indeed, when EGF signalling was analyzed in cells whose endocytosis was inhibited by a mutant dynamin, enhanced cell proliferation was observed and analysis of signal transduction components revealed hyper-phosphorylation of both PLCγ and SHC. Unexpectedly however, MAP kinase activity was significantly reduced, along with phosphorylation of ErbB-1 [63]. Although these observations are in line with some other results [137,138], some recent observations attributed the effect on MAP kinase to inhibition of MEK endocytosis [139,140]. Another possible explanation for the attenuated signalling observed in mutant dynamin expressing cells is the unexpected loss of high affinity EGF binding sites [141].

While en route to the late endosome, ErbB-1 molecules lose the ability to stimulate the PLCγ pathway, probably because components of the pathway become inaccessible [142]. Hrs is an example for a substrate of ErbB-1 whose accessibility is enhanced, rather than diminished, upon endocytosis of the receptor [110]. p21-CIP, an inhibitor of cyclin-dependent pro-

tein kinases displays a variation on the theme; its activation by EGF occurs in an endosomal compartment, but no activation occurs following exposure of cells to another ligand of ErbB-1, namely TGFα [143]. The enhanced mitogenicity of this ligand has been attributed to dissociation of ligand–receptor complexes in the endosomal compartment, which is followed by receptor recycling [144]. Interestingly, co-expression of ErbB-2 potentiates EGF signalling to the level achieved by TGFα, due to heterodimer disintegration in the early endosome, and receptor recycling to the cell surface [145,146]. Consistent with this model, formation of ErbB-1•ErbB-2 heterodimers prevents Cbl association with ErbB-1 [147].

7. Cancer therapy: harnessing the endocytic machinery?

Overexpression of ErbB-1 is frequently detected in cancers of lung, head and neck and in brain tumors (reviewed in [148,149]). In addition, a constitutively active deletion mutant of ErbB-1 is abundant in brain tumors [150] and in other types of cancers [151]. Likewise, amplification of the erbB-2 gene is associated with a relatively aggressive subtype of breast, ovary and lung cancers [152]. These clinical observations and their relationships to poor prognosis has identified ErbB proteins as attractive targets for cancer therapy, and one such agent, a humanized monoclonal antibody to ErbB-2 is already used to treat metastasizing breast cancers [153]. Importantly, in vitro studies clearly indicate that the oncogenic action of ErbB-2 depends on its localization at the plasma membrane [154,155]. Thus, removal of ErbB molecules from the cell surface by directing them to the endocytic pathway is expected to inhibit their oncogenic potential. This may be achieved by using antibodies, modified ligands, as well as agents that interfere with translocation and stabilization of the receptors at the plasma membrane.

7.1. Immunotherapy

Extensive work in animal models has indicated that certain antibodies can effectively block the tumorigenic growth of cancer cells overexpressing ErbB-1 or ErbB-2 (reviewed in [156]). The mechanism underlying the anti-tumorigenic effect of antibodies to ErbB-2 has been attributed to the recruitment of immune cells to the tumor through the Fc portion of the antibody [157]. It is important, however, that antibodies devoid of the Fc portion are active in tumor inhibition, as long as their bivalence is maintained. Several observations are consistent with the possibility that the inhibitory effect on cancer cells is due to antibody-induced removal of the oncoprotein from the cell surface: antibodies that better down regulate ErbB-2 are superior as cancer inhibitors [158]. Likewise, examination of certain combinations of anti-ErbB-2 antibodies correlated their tumor-inhibitory effect with the ability to degrade the oncogenic receptor [159]. Consistent with this scenario, antibodies to ErbB-2 and anti-TFR antibodies co-internalize through clathrin-coated pits, coated vesicles, endosomes, and MVBs [160]. The involvement of c-Cbl in antibody-induced down regulation of ErbB-2 has been addressed by mutagenesis of the putative site of Cbl binding to this receptor [161]. The mutant receptor displayed retarded antibody-induced down regulation, suggesting that tumor-inhibitory antibodies utilize, at least in part, the c-Cbl pathway to degrade ErbB-2.

7.2. Drug-induced degradation of ErbB-2

The benzoquinoid ansamycin antibiotics geldanamycin and herbimycin A were first isolated from the culture broth of several actinomycete species [162,163], and described as inhibitors of tyrosine kinase-dependent cell growth [164,165]. These compounds display inhibitory activity toward numerous cell-lines, including those over-expressing ErbB-2. This activity is attributed to the ability of geldanamycin to induce degradation of ErbB-2 and ErbB-1, as well as of other signal transducing elements [166,167]. The mechanism underlying geldanamycin-induced protein degradation, was shown to involve the dissociation of a geldanamycin binding protein, the molecular chaperone Hsp90, or in the case of ErbB-2, its family member GRP94 [168]. A complex series of proteolytic events is involved in geldanamycin-induced ErbB-2 degradation, as was evidenced by the sensitivity of inhibitors of proteasomal and lysosomal proteases [169]. By using antibodies to both extracellular and carboxyl-terminal epitopes of ErbB-2, it was shown that geldanamycin induces fragmentation of ErbB-2 within the carboxyl-terminal region of the cytoplasmic domain, and that the resulting transmembrane fragment is degraded by a mechanism that involves the formation of intracellular vesicles of membranal origin [170].

7.3. Immunotoxins

Antibodies directed against ErbB-2 may serve as useful vehicles for targeting therapeutic agents to tumors. This approach is attractive because antibodies usually internalize together with the receptor and introduce the toxic agent into the cell [158,171,172]. Conjugates of antibodies and toxins have been used in a preclinical trial as anti-tumor agents [173]. For example, a *Pseudomonas* exotoxin lacking its cell binding domain was constructed for tumor targeting [174]. Ligands directed against ErbB proteins have also been examined as beneficial carriers. For example a betacellulin–*Pseudomonas* toxin fusion is effective against cells expressing ErbB-1, but not cells expressing ErbB-4, probably due to the limited internalizing capacity of this receptor [175]. Other studies employed antibody-containing drug-loaded liposomes that efficiently bind cancer cells and deliver cytotoxic doses of doxorubicin in a targeted manner [176], probably through an ability to internalize [177].

8. Perspectives and concluding remarks

Progression into the cell cycle requires continuous ligand occupation of growth factor receptors at the cell surface for as long as 8 h. Removal of the growth factor at any step within this critical phase, abrogates subsequent commitment to S phase entry. Recent studies that are reviewed in this volume begin to reveal the molecular basis for this requirement. These include prolonged activation of Ras, up regulation of Myc, and induction of Cyclin D. However, it is already clear that endocytosis of ligand-occupied receptors, like ErbB proteins, plays a pivotal role in controlling the duration of cell activation. The ErbB family of RTKs presents a variety of mechanisms to control kinetics of signal transduction. For example, ErbB-3 evolved as a kinase-defective receptor whose signalling, as well as sorting to degradation, requires a co-receptor. On the other hand, a major function of the most oncogenic member of the family, namely ErbB-2, seems to be prolongation of signal transduction by decelerating ligand dis-

sociation, inhibiting internalization of ErbB-2-containing heterodimeric receptors, and enhancing the rate of receptor recycling. Another layer of diversity is found at the level of the ligands. Apparently, the multiple EGF- and neuregulin-like ligands differ not only in their binding specificity and affinity but also in their kinetics of signalling. The underlying mechanisms involve differential capacity to recruit the ErbB-2 co-receptor to heterodimers, as well as disparate behavior of the many ligands while they pass through sorting barriers, such as the clathrin-coated pit and the MVB.

The sorting mechanisms are expected to be resolved in the near future. It is reasonable to assume that the major players are already known. However, their sequential engagement and mutual interactions are currently unclear. It is also conceivable that many of the signalling proteins involved in signal initiation and propagation will disclose functions as players in vesicular sorting of active RTKs. Examples include cytoplasmic tyrosine kinases (e.g., Src), adapters (e.g., Grb2 and Nck), and small GTP binding proteins (e.g., Ral). Close relationships between signal initiation and the onset of signal termination are already exemplified by the sorting molecules Eps15 and c-Cbl, two early substrates of tyrosine phosphorylation. Future research will also provide an answer to the question of signalling from the endosomal compartment, an issue that remains controversial. The exact role of second messengers like calcium ions, diacylglycerol and inositol phospholipids will probably become clearer. Likewise, the extent of similarity between yeast and animal cells will have to be defined. One relevant example is the role, if any, of receptor ubiquitination as an internalization signal. Partitioning of receptors among the various endocytic pathways is another important question. Presumably, constitutive and ligand-activated pathways overlap to some extent, but molecular machineries that determine their remarkably different rates are still unknown. The relationships between these two pathways and the stress-induced endocytic response, which also involves extensive receptor endocytosis and degradation, will probably emerge.

Comprehensive understanding of the mechanisms that negatively regulate signal transduction by RTKs have wide range of implications; from embryonic development to pathological states, like wound healing, hyper-proliferative diseases and cancer. Potentially, genes involved in receptor down regulation may act as tumor suppressors. Moreover, drugs that enhance down regulation or interfere with sorting decisions are clinically useful. Indeed, humanized antibodies to ErbB-2, similar chimeric monoclonal antibodies to ErbB-1, as well as tyrosine kinase inhibitors and drugs that inhibit heat shock proteins, are already in different phases of clinical testing or application. More molecular targets for therapeutic intervention will likely emerge from future studies of receptor down regulation.

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