The interface of receptor trafficking and signalling

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Summary

The intimate relationship between receptor trafficking and signalling is beginning to reveal its secrets. Receptor endocytosis provides a mechanism for attenuation of signalling by transfer of receptors to degradative compartments. However, it can also determine signalling output by providing a different combination of downstream effectors at endocytic compartments compared with the plasma membrane. Rab5, Hrs and Cbl, are three examples of proteins that can influence both tyrosine kinase receptor trafficking and signalling pathways. By operating at this intersection, they are well placed to couple these aspects of cell function. Each element of the Rab5 GTPase cycle is influenced by signal transduction events, which will correspondingly influence recruitment of effector proteins and receptor distribution. Hrs and Cbl, which both undergo tyrosine phosphorylation in response to growth factor stimulation, are believed to influence receptor sorting in the early endosome and engage in multiple interactions, which may play a direct role in signalling cascades.

Key words: Endocytosis, Tyrosine kinase receptor, Rab5, Hrs, Hgs, EGF receptor, Cbl

Introduction

The discovery that activated EGF receptor (EGFR) is internalised by endocytosis (Haigler et al., 1979) led to speculation that this pathway provides a means for delivery of the ligand (EGF) to the nucleus, where it could directly influence gene transcription. This simple idea has garnered little supporting evidence over the years, but the discovery of cascades of signal-transducing molecules has provided us with alternative routes by which receptor activation can influence gene transcription. Endocytosis of receptors and their transport to lysosomes results in degradation of the receptors by acid-dependent proteases, which can attenuate receptor signalling and may even be conceived of as a tumour suppressor pathway (Di Fiore and Gill, 1999).

Recent work has revealed more subtle regulation of receptor signalling as a function of receptor dynamics. Subcellular localisation can determine the identity of effector molecules that couple to activated receptors and thus the relative strengths of different signalling pathways.

EGFR typically recycles through the sorting or early endosome to the plasma membrane an estimated 3-5 times before it is selected for degradation in a stochastic manner by routing to late endosomal compartments. At steady state, 70-80% of the EGF-occupied receptor is endosomal (Sorkin, 1998). Several lines of evidence suggest that the receptor can maintain its activated state during a substantial part of this cycle. The ability of the receptor to autophosphorylate is maintained (Lai et al., 1989), and various signalling molecules, such as Shc, Grb2 and mSOS, redistribute to early endosomes in a ligand-dependent manner (Di Guglielmo et al., 1994; Oskvold et al., 2000). One is led to the conclusion that the majority of EGF-dependent receptor signalling actually occurs from endosomal compartments. Even after normalisation for receptor density, the specific activity of some receptor signalling events will be enriched on endosomes simply by a temporal coincidence – the rate at which signals pass down a pathway versus the receptor internalisation rate. But can location actually determine or specify signalling outcomes? Very probably. Vieira et al. have examined the influence of endocytic trafficking on EGF-dependent signalling after imposing a block on EGFR endocytosis by expression of a dominant negative form of dynamin (Vieria et al., 1996). They found that the mutant dynamin changed the relative strength of EGF-dependent signalling pathways; for example, Erk1 phosphorylation dramatically decreased whereas phospholipase Cγ phosphorylation increased.

If membrane trafficking can influence signal transduction, then it should be no surprise if the reverse is true – nature loves a feedback loop after all. For coupling to occur, a factor must engage with both processes. Here, we highlight three examples of proteins that can regulate aspects of both EGFR trafficking and signalling.

Rab5

Rab proteins are a family of small GTPases with firmly established roles in membrane traffic. They regulate SNARE complex assembly preceding vesicle fusion, at least in part by recruiting tethering factors, such as early endosomal autoantigen 1 (EEA1), which is recruited by Rab5 (Clague, 1999). Rab5 is localised to the early endosome, where it interacts with multiple effector molecules and regulates both endosome fusion and motility (Gorvel et al., 1991; Nielsen et al., 1999). It has also been proposed to regulate endocytic clathrin-coated vesicle formation (Bucci et al., 1992; McLauchlan et al., 1998).

It has long been known that EGF stimulates not only receptor downregulation but also fluid-phase internalisation. Barbieri et al. have recently shown that both forms of stimulated endocytosis are Rab5 dependent and coupled to

Rab5 activation (Barbieri et al., 2000). Addition of EGF to serum-starved cells leads to GTP loading of Rab5. This pathway might involve PI 3-kinase and protein kinase B (PKB/Akt), because wortmannin and expression of dominant negative PKB/Akt block the stimulation of endocytosis by EGF (Barbieri et al., 2000). Interestingly, Christoforidis et al. have shown that Rab5 interacts with two PI 3-kinases, hVPS34 and p110β, using affinity chromatography with a Rab5-GTP column (Christoforidis et al., 1999). The hVPS34 enzyme (a class III PI 3-kinase, the human homologue of Vps34p – the only PI 3-kinase in yeast) generates phosphatidylinositol 3-phosphate (PtdIns(3)*P*), which in turn cooperates with Rab5 itself to recruit EEA1 to endosomes, where it is believed to participate in endosome fusion events (Mills et al., 1998; Simonsen et al., 1998). The interaction between Rab5 and p110β PI 3-kinase (a class I PI 3-kinase) suggests that Rab5 may play a role in the localised production of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 and consequent activity of downstream effectors (e.g. PKB). Among other outputs, this may provide a positive feedback loop by stimulating further activation of Rab5 (Barbieri et al., 1998).

Lanzetti et al. have recently uncovered a mechanism that might limit the stimulatory effects of EGF described above. Eps8 is a substrate for the EGF receptor kinase and can recruit RN-tre (**r**elated to the **N**-terminus of TRE; (Matoskova et al., 1996b) to the plasma membrane through its SH3 domain (Matoskova et al., 1996a). Lanzetti et al. have shown that RN-tre can act as a Rab5 GAP and thereby inactivate Rab5 by stimulating its GTP hydrolysis. Indeed over-expression of RN-tre leads to reduction of both EGF and transferrin endocytosis, except in Eps8-null fibroblasts, in which only transferrin uptake is inhibited (Lanzetti et al., 2000). A further consequence of RN-tre expression is inhibition of Eps8-dependent Rac activation: RN-tre competes for Eps8 binding with E3bl, an adapter protein that recruits Sos-1, an exchange factor for Rac (Scita et al., 1999). Thus, inhibition of EGFR endocytosis and Rac signalling are coupled through Eps8-dependent recruitment of RN-tre (Lanzetti et al., 2000).

A key component of the Rab5 cycle is Rab-GDI, which sequesters multiple Rab proteins away from membranes in their GDP-bound form and chaperones them within the cytosol (Ullrich et al., 1993). The observation that GDI is phosphorylated led to the suggestion that this provides a signal for coordinated regulation of Rab family activity (Steele-Mortimer et al., 1993). Cavalli et al. have recently identified a GDI-activating factor as p38 MAP kinase, which phosphorylates GDI and thereby causes extraction of Rab5 from membranes (Cavalli et al., 2001). These authors linked the p38-induced stress response following peroxide treatment or UV illumination to an increase in the endocytic rate constant that correlates with p38-dependent promotion of cytosolic GDI-Rab5 complex formation. At first this result seems counterintuitive, because Rab5 is active at membranes, but the authors argue that it reflects the increased turnover of Rab5, which is known to be linked to nucleotide exchange. Note that in BHK cells up to 80% of membrane-bound Rab5 is in the inactive GDP-bound form (Stenmark et al., 1994). Cytosolic Rab5-GDI may therefore be limiting in endocytic transport, as has been suggested by studies of clathrin-coated vesicle formation in a permeabilised cell assay (McLauchlan et al., 1998). These studies have revealed a hitherto unsuspected aspect of the stress-response programme: an acceleration of endocytic rate that is likely to be mediated by p38-dependent phosphorylation of GDI (Cavalli et al., 2001).

The recent data described above point to a central role for the endocytosis regulator Rab5 in coordinating the response to various stimuli. Three aspects of the Rab5 cycle – GTP exchange, GTP hydrolysis and GDI sequestration – thus intersect with classical cell signalling pathways (Fig. 1). Rab 5 may translate these signals by influencing receptor localisation and hence signalling output as discussed above, but may also directly influence signalling by recruitment of effector proteins (e.g. PI 3-kinase enzymes) to endosomes.

Hrs

Hepatocyte growth factor **r**egulated tyrosine kinase **s**ubstrate (Hrs, also called Hgs) is an endosome-localised protein that is a prominent target for phosphorylation downstream of a variety of receptors, including Met, EGFR, and the receptors for GM-CSF and interleukin 2 (reviewed by Komada and Kitamura, 2001). It bears significant similarity (including a FYVE-finger motif and a VHS domain) to the yeast protein Vps27p. Vps27p belongs to the class E Vps mutants, which exhibit defective transport from the sorting endosome to the vacuole (Bankaitis et al., 1986; Raymond et al., 1992).

Internalisation of EGFR to Hrs-containing endosomes is required for EGF-induced Hrs phosphorylation (Urbé et al., 2000). This phosphorylation event requires PI 3-kinase

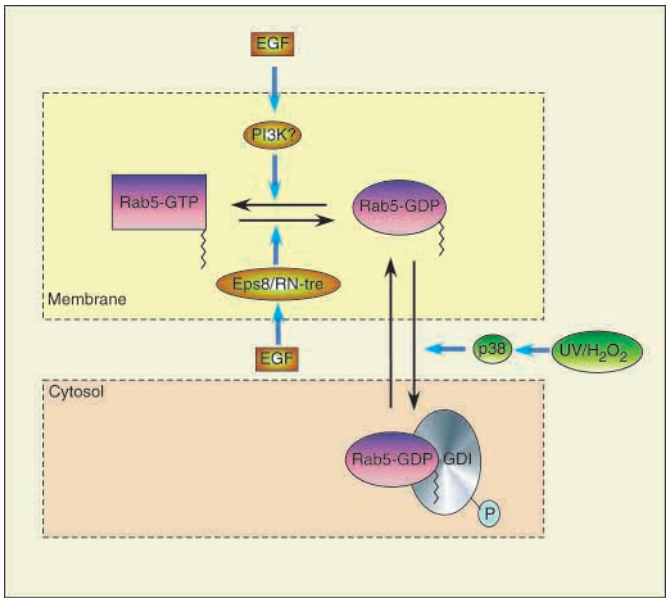

Fig. 1. Signal transduction and the Rab5 cycle. EGF stimulation leads to opposing effects on Rab5 nucleotide status. One pathway leads to an increase in active GTP-bound Rab5 and receptor internalisation through stimulation of nucleotide exchange, possibly through PI 3-kinase activation. Conversly, EGF stimulation can also negatively regulate endocytosis by activating the GTPase activity of Rab5 through the sequential recruitment of Eps8 and RN-tre to the receptor. Stimulation of endocytosis is also observed in response to p38 MAP-kinase activation by UV or peroxide (H_2O_2). This effect is proposed to be mediated through phosphorylation and activation of GDI, a chaperone of cytosolic GDP-bound Rab proteins, which results in a net increase in Rab5 cycling.

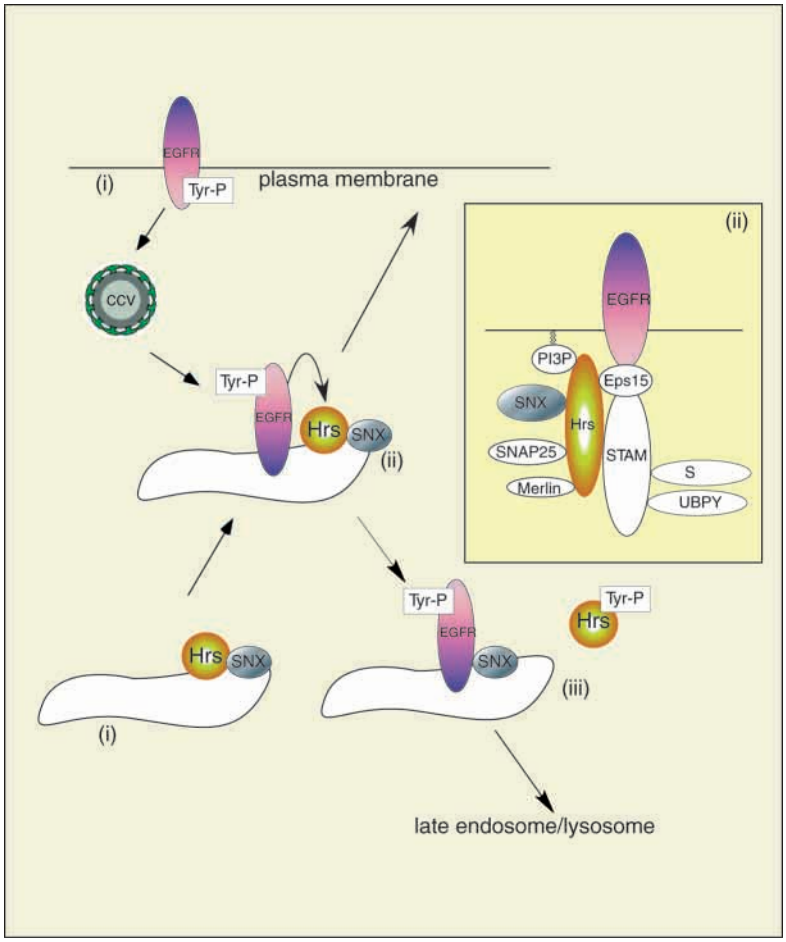

Fig. 2. Hrs is an adapter molecule implicated in endosomal sorting. Proposed mechanism by which Hrs mediates downregulation of EGFR by recruiting sorting nexin 1 (SNX). (i) EGF binding induces tyrosine phosphorylation of EGFR, causing its internalisation by clathrin-coated vesicles (CCV) to an early or sorting endosome. Hrs is recruited to the early endosome through PtdIns(3)*P*-binding to its FYVE domain and associates with endosomal sorting nexin. (ii) The coincident translocation of the EGFR from the plasma membrane and Hrs from the cytosol to the endosome results in the tyrosine phosphorylation of Hrs. This in turn leads to the dissociation of the tyrosine-phosphorylated Hrs from the membrane and we speculate may liberate the sorting nexin to bind the EGFR and route it to the late endosome and lysosome for degradation. The insert shows a simplified illustration of the adapter function of Hrs. Hrs binding to the membrane is partially dependent on PtdIns(3)*P* interaction with its FYVE domain. Hrs is linked to the EGFR by interaction of a proline-rich domain with Eps15, which also binds to EAST, a STAM/Hbp homologue. SNX1 binds to a region encompassing the proline-rich domain and the coiled-coil domain of Hrs. The major coiled-coil domain of Hrs interacts with STAM and Hbp, as well as with SNAP25. Hrs also interacts with the NF2 gene product, Schwannomin (also known as Merlin). STAM/Hbp in turn recruits the protein AMSH and the de-ubiquitination enzyme UBPY through an SH3 domain. For detailed domain structures see Komada and Kitamura, 2001 (Komada and Kitamura, 2001).

activity, as evidenced by its sensitivity to wortmannin. This is at least in part due to interaction of PtdIns(3)*P* with the FYVE domain, which contributes to the localisation of Hrs on endosomes. Interestingly, once phosphorylated, Hrs seems to be released from the endosome membrane to the cytosolic pool (Urbé et al., 2000).

Two proteins that bind to Hrs have been identified: signal-transducing adapter molecule (STAM) and Hrs-binding protein (Hbp), the mouse homologue of human STAM-2 (Asao et al., 1997; Endo et al., 2000; Takata et al., 2000). Human STAM and STAM-2 share 57% sequence identity, each bears an SH3 domain and both are also tyrosine phosphorylated. In T cells, overexpression of Hrs leads to suppression of cytokine-mediated DNA synthesis, whereas overexpression of a mutant unable to bind STAM has no effect (Asao et al., 1997). A highly related chicken protein, EAST (EGF-receptor-associated protein with SH3 and TAM domains), which associates with EGFR and Eps15, is also phosphorylated after EGF stimulation (Lohi et al., 1998). *Saccharomyces cerevisiae* contains a single Hbp/STAM homologue, YHL002W, which has been shown to interact with the Hrs homologue Vps27p in a systematic two-hybrid screen (Uetz et al., 2000).

NIH3T3 cells stably transfected with Hbp mutants that lack the SH3 domain or the binding site for Hrs exhibit impaired degradation of internalised PDGF (Takata et al., 2000). This latter result is consistent with a role for the Hrs-Hbp complex in regulating transport from early to late endosomes (or multi-vesicular bodies) proposed by analogy with Vps27p function in yeast.

A two-hybrid screen revealed a further interesting partner for Hrs, the rat homologue of human sorting nexin 1 (SNX1; Chin et al., 2001), which shares with Hrs the ability to bind to PtdIns3*P*, (although SNX1 utilises a PX domain rather than a FYVE domain; Wishart et al., 2001). SNX1 also interacts directly with the lysosomal sorting signal of EGFR, and its overexpression leads to increased receptor degradation (Kurten et al., 1996). There is an overlap between the EGFR- and Hrs-binding sites of SNX1, and the interactions appear to be mutually exclusive (Chin et al., 2001). This suggests Hrs might act as a negative regulator of EGFR degradation by sequestering SNX1 at the membrane. The observation that overexpression of Hrs, or more crucially the SNX1-binding domain of Hrs, inhibits EGFR degradation in HeLa cells supports such a model (Chin et al., 2001). The Hrs-SNX1 complex is found only in particulate fractions, whereas each protein exists in a separate cytosolic pool. Perhaps phosphorylation of Hrs, which we have proposed leads to its release from the membrane (Urbé et al., 2000), provides the trigger for the dissociation of the Hrs-SNX1 complex. This model is attractive because it provides a mechanism for the receptor to govern its own fate at the endosome by coupling the receptor-mediated phosphorylation of Hrs-Hbp to availability of a sorting factor (Fig. 2).

Kato et al. recently employed a far-western screen to identify

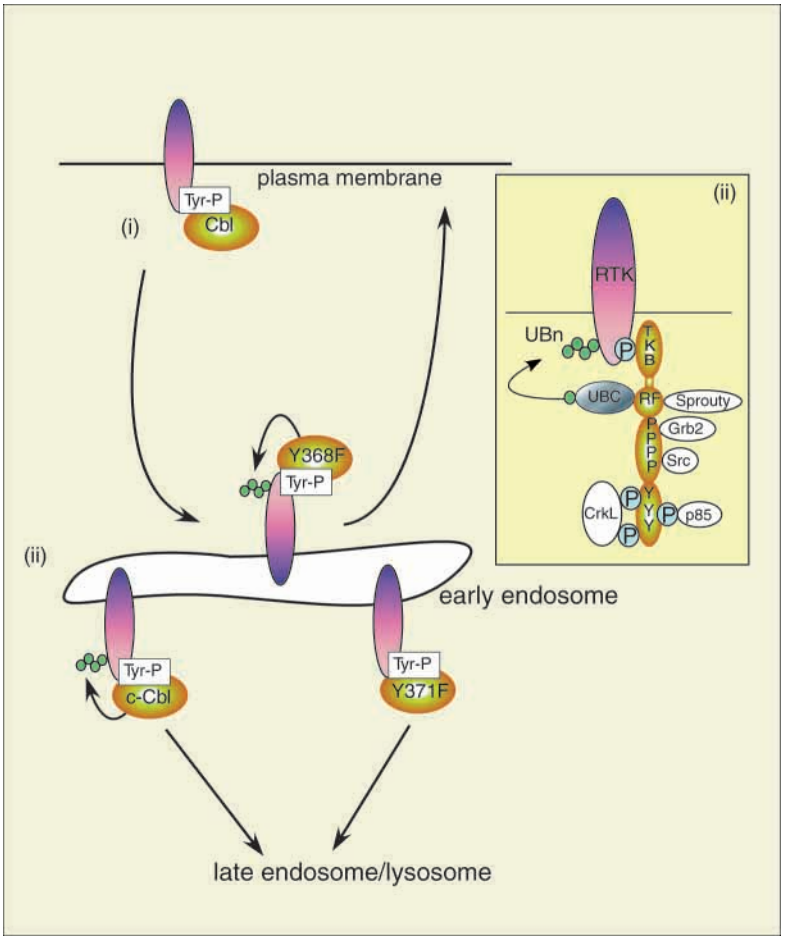

Fig. 3. Cbl, an E3 ubiquitin ligase, regulates receptor sorting and acts as an adapter protein. Schematic illustration of the differential effects on polyubiqitination and sorting of receptor tyrosine kinases (RTKs) of two Cbl mutants. (i) Ligand-induced tyrosine phosphorylation (Tyr-P) of the RTK leads to the binding of Cbl to the receptor and internalisation via clathrin-coated vesicles to the early or sorting endosome. (ii) Wild-type Cbl (c-Cbl) mediates the polyubiquitination of the receptor tail through recruitment of ubiquitin-conjugating enzymes (UBCs or E2 ligases, see insert) and concomitant sorting of the receptor to late-endosomal/lysosomal compartments where degradation takes place. The Y371F linker mutant of Cbl does not mediate polyubiquitination but can govern sorting of the receptor for degradation. The nearby Y368F linker mutation produces a Cbl mutant able to mediate polyubiquitination but incapable of re-routing the receptor from the plasma-membrane-directed recycling pathway to the lysosome. The insert shows a simplified illustration of the E3-ligase and adapter functions of Cbl. Cbl binds to the tyrosine phosphorylated (P) receptor (RTK) through its tyrosine-kinase-binding domain (TKB), which is connected to the RING-finger domain (RF) through an essential linker. The RING-finger domain recruits ubiquitin-conjugating enzymes (UBCs), which transfer ubiquitin (Ubn) onto the receptor. The RF also binds Sprouty, whereas the proline-rich domain (PPPP) binds the adapter Grb2 and Src kinase. The C-terminal domain recruits CrkL and p85-p110 PI3-kinase through phosphorylated tyrosines Y700/Y774 and Y731, respectively. For a more complete list of interactions, see Thien and Langdon, 2001 (Thien and Langdon, 2001).

a deubiquitinating enzyme, UBPY, as an Hbp/STAM-2 binding partner (Kato et al., 2000). Although no functional data concerning this interaction are currently available, this observation is intriguing because of the extra possibilities it raises about Hrs regulation of receptor trafficking. As we discuss below, receptor ubiquitination could be a key determinant of receptor sorting. Indeed Hrs, STAM, Hbp/STAM-2 as well as two other endocytic proteins, epsin and Eps15, have been proposed to possess a ubiquitin-interaction motif (UIM; Hofmann and Falquet, 2001), the significance of which remains to be elucidated.

Hrs function is thus clearly multifaceted. By virtue of multiple interactions that are depicted in Fig. 2, it is able both to regulate receptor trafficking and to participate in signal transduction pathways.

Cbl

Studies of *Caenorhabditis elegans* vulval development led to the identification of SLI-1 (suppressor of lineage defect), a Cbl homologue, as a negative regulator of signalling downstream of the LET-23 tyrosine kinase receptor, which is a homologue of the mammalian EGFR (Yoon et al., 1995). It has since been proposed that the principal way in which Cbl acts as a negative regulator of tyrosine kinase signalling is by promoting receptor downregulation (Lee et al., 1999; Levkowitz et al., 1998; Miyake et al., 1998). Cbl acts as an E3 ubiquitin ligase, bringing together phosphorylated receptors and E2 ubiquitin-conjugating enzymes by interactions with a tyrosine-kinase-binding (TKB) domain (Lupher et al., 1999) and a RING-finger domain (Freemont, 2000), respectively (reviewed by Thien and Langdon, 2001). This results in receptor polyubiquitination, which rather than specifying proteasomal degradation appears to enhance transport to late-endosome/lysosomal compartments in which degradation occurs (Bonifacino and Weissman, 1998). A Cbl 1-440 construct, which contains both the TKB domain and the RING-finger domain but lacks the C-terminal region that interacts with a variety of other factors (see below and Fig. 3), behaves the same as the wild-type protein in promoting the downregulation of surface EGFR from HEK293 cells (Lill et al., 2000).

Where does Cbl-dependent ubiquitination of receptors occur? Levkowitz et al. have shown that expression of Cbl, or mutants that influence EGFR degradation, does not influence receptor internalisation kinetics when receptors are expressed at high levels in CHO cells (Levkowitz et al., 1998). Co-expression of the dominant negative v-Cbl, which lacks the C-terminal half including the RING-finger domain, antagonises receptor degradation by competing with Cbl for receptor binding. Following co-expression of v-Cbl and Cbl, receptors recycle normally. This antagonistic effect of v-Cbl towards Cbl is abrogated by treatment of cells with monensin, perhaps because monensin treatment can itself inhibit the receptor recycling pathway (Levkowitz et al., 1998). These results,

together with the observation that EGF promotes a transient colocalisation of Cbl and receptor in internal vesicles, led Levkowitz et al. to favour an endosomal site of action for Cbl and consequent inhibition of receptor recycling. Note that Hrs/Hbp/UBPY, located at the early endosome, would be expected to antagonise Cbl function (see above). However, another study, which has not directly looked at Cbl involvement, has shown that, following stimulation, EGFR is ubiquitinated at the plasma membrane of HeLa cells (Stang et al., 2000). Stang et al. imposed a block on endocytosis by expressing dominant negative dynamin. This block did not affect the efficiency of receptor ubiquitination. A model consistent with both studies would be for Cbl-mediated receptor ubiquitination to occur at the plasma membrane, but for its principal effect to be exerted at the early endosome by antagonising receptor recycling. This would also fit with recent observations of de Melker et al. who observe EGF-dependent translocation of Cbl to the plasma membrane in transfected COS cells (de Melker et al., 2001). However, Burke et al. have developed immunoprecipitation protocols that show that activated EGFR acquires endogenous Cbl as it traffics through intracellular compartments of a mammary epithelial cell line (Burke et al., 2001), which has ensured that this issue remains controversial.

Phosphorylation of Y1045 of the EGFR is required for Cbl recruitment, and its mutation blocks receptor ubiquitination and downregulation (Levkowitz et al., 1999). However, a recent paper has challenged a causative relationship between receptor ubiquitination and Cbl-dependent downregulation (Thien et al., 2001; see Fig. 3). A Y368F-Cbl linker region mutant is as effective as wild-type Cbl in promoting EGFR ubiquitination but does not have an equivalent ability to inhibit receptor recycling when expressed in NIH3T3 cells. Conversely, a Y371F-Cbl mutant is unable to promote receptor polyubiquitination and yet resembles wild-type Cbl in its inhibition of receptor recycling (Thien et al., 2001). One must therefore consider the possibility that targets for ubiquitination other than the receptor itself have an influence on receptor sorting. Alternatively, Cbl might act through a ubiquitin-independent mechanism to promote receptor degradation. A caveat to this challenging study is that the measured amount of recycling receptor was small in the control cells (NIH3T3 cells expressing relatively low amounts of receptor; Thien et al., 2001).

There are many other aspects to Cbl function beyond its role as an E3 ligase (reviewed by Thien and Langdon, 2001). Disruptions of the RING-finger domain abolish this activity but are insufficient to convert Cbl to an oncogenic protein (Thien et al., 2001). The critical region for oncogenesis appears to be an α-helical linker domain that connects the RING finger to the SH2 region of the TKB domain (Thien et al., 2001). The RING-finger domain of Cbl can also interact with Sprouty, an antagonist of tyrosine kinase receptor signalling (Wong et al., 2001). Cbl itself is a substrate for tyrosine kinases and may function as an adapter protein. Phosphorylation of Y700 and Y774 provides a docking site for the adapter protein CrkL, which can lead to JNK activation downstream of Met receptor stimulation (Garcia-Guzman et al., 2000). Phosphorylated Y731 provides a docking site for the p85 PI 3-kinase adapter subunit (Hartley and Corvera, 1996). Furthermore, Cbl apparently exerts a positive regulatory role in integrin signaling downstream of Src by recruiting both PI 3-kinase and CrkL (Feshchenko et al., 1999).

The details of the influence of Cbl on cellular signalling remain to be fully explored, but all the signs are that it can combine regulation of receptor trafficking and signalling, in part by functioning as an adapter protein.

Conclusions

The term scaffold or adapter protein, in the context of signal transduction, connotes a protein that brings together modular components of a pathway or coordinates elements of separate pathways. We would like to propose consideration of a further class of adapter protein, which coordinate receptor signalling and trafficking. We have highlighted three proteins (among several other likely candidates, including β-arrestin, a regulator of G-protein coupled receptor transport; Luttrell et al., 1999) that provide pertinent examples. Each of the three proteins discussed above, Rab5, Hrs and Cbl, has been clearly implicated in regulation of tyrosine kinase receptor trafficking. By this measure alone, they will influence receptor signalling, but each is also likely to be directly involved in signal transduction cascades that are regulated by EGFR. In the case of Rab5, the nucleotide state (GDP or GTP) is sensitive to EGF stimulation, whereas Hrs and Cbl are both tyrosine phosphorylated. These changes provide each with the opportunity to respond to EGF stimulation by modifying associations with effector proteins that may in turn regulate receptor trafficking and/or signalling. Many outstanding issues specific to each protein remain. For example, stimulation of EGFR can unleash both Rab5-activating and -inactivating activities. The timing and locations of these two outcomes will need to be elucidated. What is the significance of Hrs and Hbp phosphorylation? And, finally, although ubiquitination clearly plays a role in the endocytic pathway, could receptor ubiquitination turn out to be a red herring with regards to the mechanism of Cbl-dependent receptor degradation?

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