Spatial and Temporal Regulation of Receptor Tyrosine Kinase Activation and Intracellular Signal Transduction

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EGF, insulin, signaling endosome, membrane trafficking, ligand degradation, signaling interactome

Abstract

Epidermal growth factor (EGF) and insulin receptor tyrosine kinases (RTKs) exemplify how receptor location is coupled to signal transduction. Extracellular binding of ligands to these RTKs triggers their concentration into vesicles that bud off from the cell surface to generate intracellular signaling endosomes. On the exposed cytosolic surface of these endosomes, RTK autophosphorylation selects the downstream signaling proteins and lipids to effect growth factor and polypeptide hormone action. This selection is followed by the recruitment of protein tyrosine phosphatases that inactivate the RTKs and deliver them by membrane fusion and fission to late endosomes. Coincidentally, proteinases inside the endosome cleave the EGF and insulin ligands. Subsequent inward budding of the endosomal membrane generates multivesicular endosomes. Fusion with lysosomes then results in RTK degradation and downregulation. Through the spatial positioning of RTKs in target cells for EGF and insulin action, the temporal extent of signaling, attenuation, and downregulation is regulated.

Contents

INTRODUCTION

As exemplified by the epidermal growth factor (EGF) receptor tyrosine kinase (RTK) (**Figure 1**), a stepwise delivery of ligand-associated receptors from the cell surface to intracellular vesicles of the endosomal apparatus coordinates the extent of growth factor signaling. Through recent advances in the techniques of quantitative imaging and mass spectrometry, the spatiotemporal details of RTK signaling, attenuation, and downregulation have been resolved.

Within seconds, EGF association with its RTK on the cell surface generates dimeric EGF–RTKs. Dimerization activates RTK tyrosine autophosphorylation and the recruitment of associated signaling proteins. This activation coincides with the budding of clathrin-coated vesicles enriched in the RTKs, followed by rapid formation of free clathrin-coated vesicles. As the coat disassembles, the RTK-enriched vesicles associate with actin and move along microtubules into the cell interior. Vesicle generation from the cell surface may also operate through nonclathrin pathways, but such vesicles still deliver activated RTKs to early endosomes by membrane fusion.

Peak concentration of activated RTKs in early endosomes is observed in minutes with receptor autophosphorylation, recruitment of downstream signaling proteins, tyrosine phosphorylation, and signal transduction to the cell interior. These early endosomes are thus signaling endosomes whose numbers increase as higher doses of extracellular EGF engage more cell surface RTKs and more endocytosis. Membrane fusion of endosomes triggers the recruitment of phosphotyrosine phosphatase(s) that dephosphorylate the RTKs. After further membrane fission, dephosphorylated RTKs segregate to late endosomes. These late endosomes also degrade any intraendosomal EGF ligands through proteinase cleavage of EGF by cathepsin B. Further fission mediated by the endoplasmic reticulum (ER) generates recycling endosomes that return deactivated RTKs to the cell surface. Any RTKs remaining in the late endosome are covalently modified by ubiquitinylation

EGF: epidermal growth factor

RTK: receptor tyrosine kinase

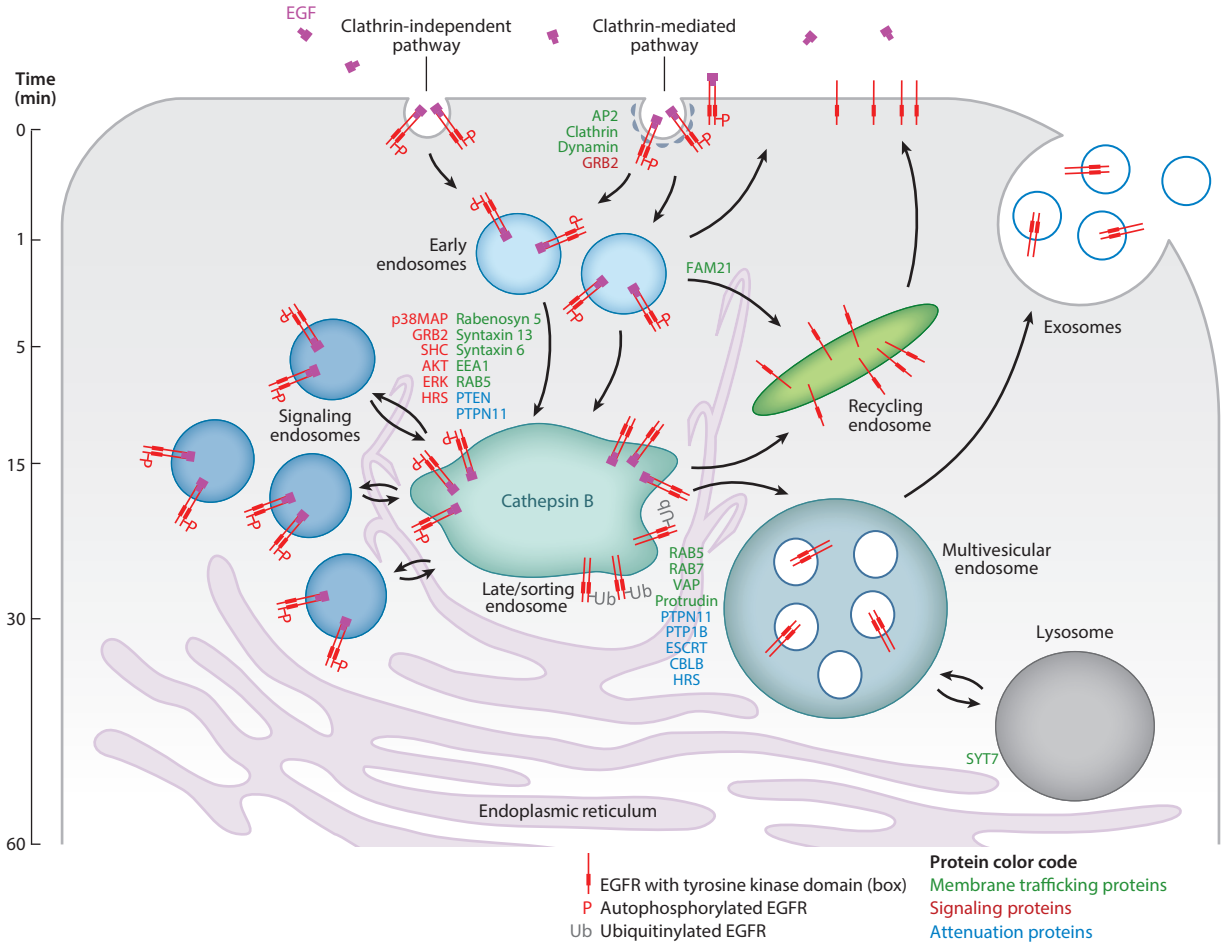

Figure 1

Overview of membrane trafficking and RTK signaling. Ligand association of EGF–RTK concentrates dimerized, activated RTK in clathrin-coated and non-clathrin-coated pits with immediate internalization into early endosomes that become signaling endosomes after membrane fusion. Coincidental to the recruitment of phosphotyrosine phosphatases, the endosomal fusion machinery in cooperation with the endoplasmic reticulum–mediated membrane fission machineries (involving FAM21, VAP, and SYT7; see Reference 57) sequesters dephosphorylated receptors to be recycled back to the plasma membrane or targeted for degradation. Recycling endosomes are sorted away from the late endosome as it matures to a multivesicular endosome coincidental to EGF–RTK ubiquitinylation, recruitment of ESCRT proteins, and fusion with lysosomes that results in EGF–RTK degradation. A proportion of multivesicular endosomes may fuse with the surface membrane to generate exosomes. Trafficking, signaling, and attenuation proteins are indicated in different colors (see key). Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ESCRT, endosomal sorting complexes required for transport; FAM21, family with sequence similarity 21; RTK, receptor tyrosine kinase; SYT7, synaptotagmin-7; VAP, VAMP-associated protein.

through c-Cbl (c-Casitas B-lineage lymphoma proto-oncogene, E3 ubiquitin protein ligase). RTK ubiquitinylation recruits endosomal sorting complexes required for transport (ESCRT) proteins that deform the late endosomal membrane to form inwardly budding membranes that pinch off to form free intralumenal vesicles. In these multivesicular endosomes, a two-membrane barrier now exists between the signaling domain of the receptor and the cytoplasm. Subsequent fusion of

the multivesicular endosome with lysosomes leads to receptor degradation and, hence, receptor downregulation.

In summary, signal propagation occurs in signaling endosomes, whereas receptor desensitization takes place in larger late endosomes in which phosphotyrosine phosphatase recruitment generates inactivated receptors. The proportion of RTKs recycling to the surface or remaining in the late endosome as it matures to the multivesicular endosome and lysosome for RTK degradation is dependent on RTK concentration in the late endosome. A further sorting event occurs as the multivesicular endosome fuses with the surface membrane, leading to discharge of the intralumenal vesicles to the extracellular environment as exosomes. These exosomes retain the intralumenal sequestration of the tyrosine kinase while exposing the ligand-binding domain of the receptor to the extracellular milieu.

High-resolution and high-dynamic-range tandem mass spectrometry have been used to quantitatively characterize receptor phosphorylation, ubiquitinylation, and association with signaling and attenuation proteins. This strategy has also contributed to the spatiotemporal mapping of endosomal subcompartments engaged in RTK traffic and the regulation of signal transduction. X-ray crystallography and eventually cryo–electron microscopy (cryo-EM) visualization of activated and inactivated RTK with downstream scaffolds, signaling, and attenuation proteins will soon uncover new mechanisms to coordinate membrane traffic with signal transduction.

INSULIN AND EGF ACTION IN LIVER

The liver is the major organ that regulates the impact of insulin on metabolic homeostasis (1). Insulin regulates glucose availability in the circulatory system via the insulin-mediated reversible storage of glucose as glycogen and increased lipogenesis through well-established insulin signaling pathways (e.g., 2, 3). The liver is one of the few organs capable of regeneration following liver damage or removal of up to 75% of its mass. EGF has been strongly implicated in the regulation of this growth and development following liver injury (4, 5) via well-described EGF signaling pathways (e.g., 6).

Early observations on the spatial and temporal aspects of insulin and EGF signaling were first demonstrated in liver parenchyma (**Figure 2**). Insulin was shown to associate with the cell surface of hepatocytes followed by rapid entry into nonlysosomal lipoprotein-filled structures (7, 8). Results from subcellular fractionation studies and the EM localization of radiolabeled insulin in the isolated fractions suggested an uptake into Golgi vesicles containing newly synthesized intralumenal lipoproteins (9, 10) (**Figure 2*e***, insets). However, these vesicles were endosomes because their lipoprotein content represented internalized lipoproteins (11) (**Figure 2*f***).

The time course of insulin receptor tyrosine kinase (insulin-RTK) activation (i.e., tyrosine autophosphorylation of the insulin-RTK and augmentation of its capacity to phosphorylate substrates) revealed its rapid entry and high concentration in endosomes as an identified locus of signal transduction in vivo (12). The key determinant of insulin signaling is the tyrosine phosphorylation status of the insulin-RTK, as peroxovanadium compounds (potent tyrosine phosphatase inhibitors) activated the insulin-RTK in the absence of insulin (13). Furthermore, the administration of peroxovanadium compounds in conditions in which the hepatic endosomal insulin kinase was selectively activated resulted in insulin signaling (14).

Parallel studies with EGF and its cognate receptor demonstrated a similar route of internalization and compartmentalization in liver and other target cells (15–21) (**Figure 2*a–d***). Following EGF administration in vivo, the time course of EGF–RTK internalization, its activation and autophosphorylation in endosomes, and its recycling to the plasma membrane were demonstrated as well as an augmented tyrosine phosphorylation of its substrate Src homology 2–containing protein

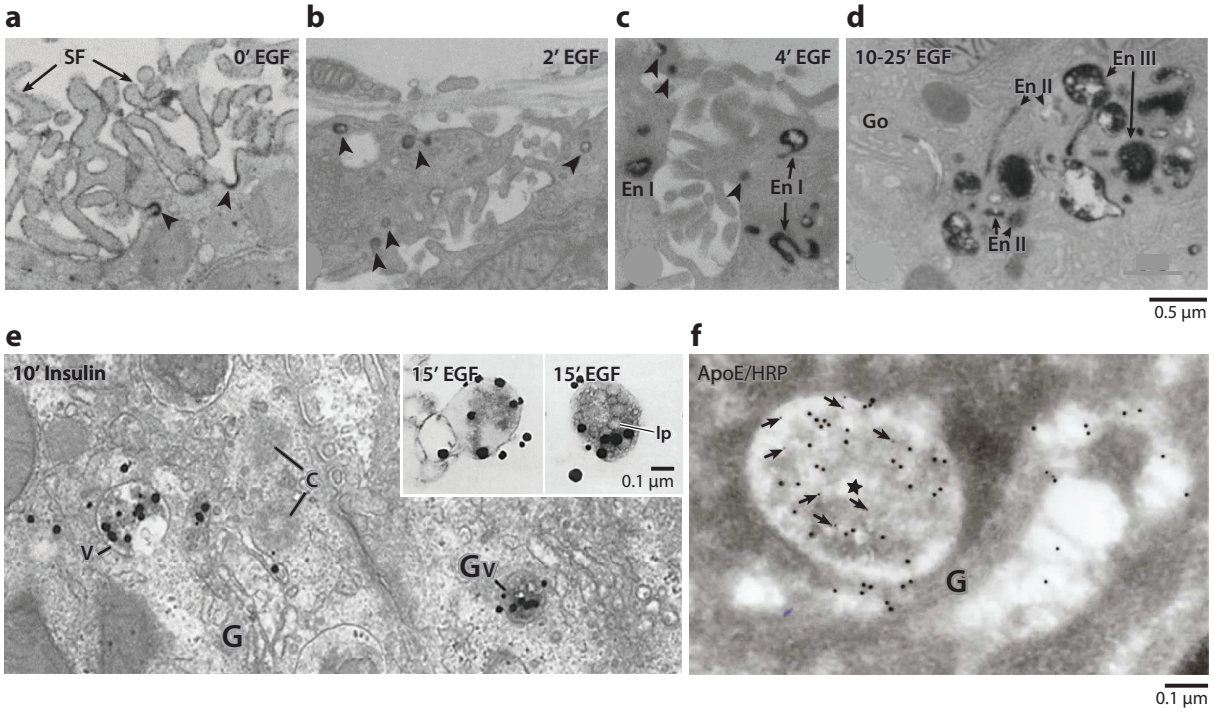

Figure 2

Morphology of EGF and insulin internalization compartments in liver. (*a*) EGF association at 4°C with the SF of the liver plasma membrane and concentration in coated pits (*arrowheads*) are seen through the reaction product of EGF coupled to horseradish peroxidase. (*b–d*) EGF at 2, 4, and 10–25 min, respectively, after perfusion at 35°C showing uptake into endosomes. Panels *a–d* adapted from Reference 18 with permission. ©1984 Dunn & Hubbard. *J. Cell Bio.* 98:2148–59. doi:10.1083/jcb.98.6.2148. (*e*) ^{125}I-insulin uptake by liver at 10 min as visualized by electron microscope quantitative radioautography, revealing concentration in vesicles (v) next to stacked cisternae of the Golgi apparatus (G). Panel *e* insets: ^{125}I-EGF localization in liver endosomes isolated at 15 min after EGF administration. Panel *e* adapted from Reference 8 with permission. ©1979 Bergeron et al. *J. Cell Bio.* 80:427–43. doi:10.1083/jcb.80.2.427. Panel *e* inset adapted from Reference 24 with permission. ©1989 Lai et al. *J. Cell Bio.* 109:2751–60. doi:10.1083/jcb.109.6.2751. (*f*) Endosomal colocalization of apoE (10-nm gold) and 10-min internalized horseradish peroxidase (5-nm gold, *arrows*) in liver cryosections. Golgi apparatus reveals only 10-nm gold apoE immunoreactivity (the *star* represents large endocytic components closely opposed to the Golgi apparatus). Panel *f* adapted from Reference 11 with permission. ©1994 Dahan et al. *J. Cell Bio.* 127:1859–69. doi:10.1083/jcb.127.6.1859. Abbreviations: apoE, apolipoprotein E; EGF, epidermal growth factor; EnI, large vesicles and tubules; EnII, small and large vesicles and tubules in the Golgi-lysosome region; EnIII, lipoprotein-containing vesicles or multivesicular bodies; lp, lipoprotein; SF, sinusoidal front.

(SHC) on the cytosolic surface of endosomes (22–26) (**Figure 3**). The fates of both insulin and EGF ligands in liver parenchyma were followed: The sites of initial cleavage of the insulin and EGF polypeptides and the cognate enzymes were uncovered to be endosomal (27–31).

Today, imaging in live cells (**Figure 4**) has defined with exquisite precision the time course of EGF binding to its RTK in model cell lines and the time course of RTK concentration in endosomes, their autophosphorylation and recruitment of EGF–RTK substrates, and their dephosphorylation. Furthermore, a detailed molecular dissection for the proteins linked to every step in EGF–RTK endocytosis and a quantitative assessment of EGF–RTK signaling at each locus in the endocytic pathway have been described. Endosomes are deduced to be a major site for EGF signal transduction and the site of recruitment of the tyrosine phosphatases that regulate

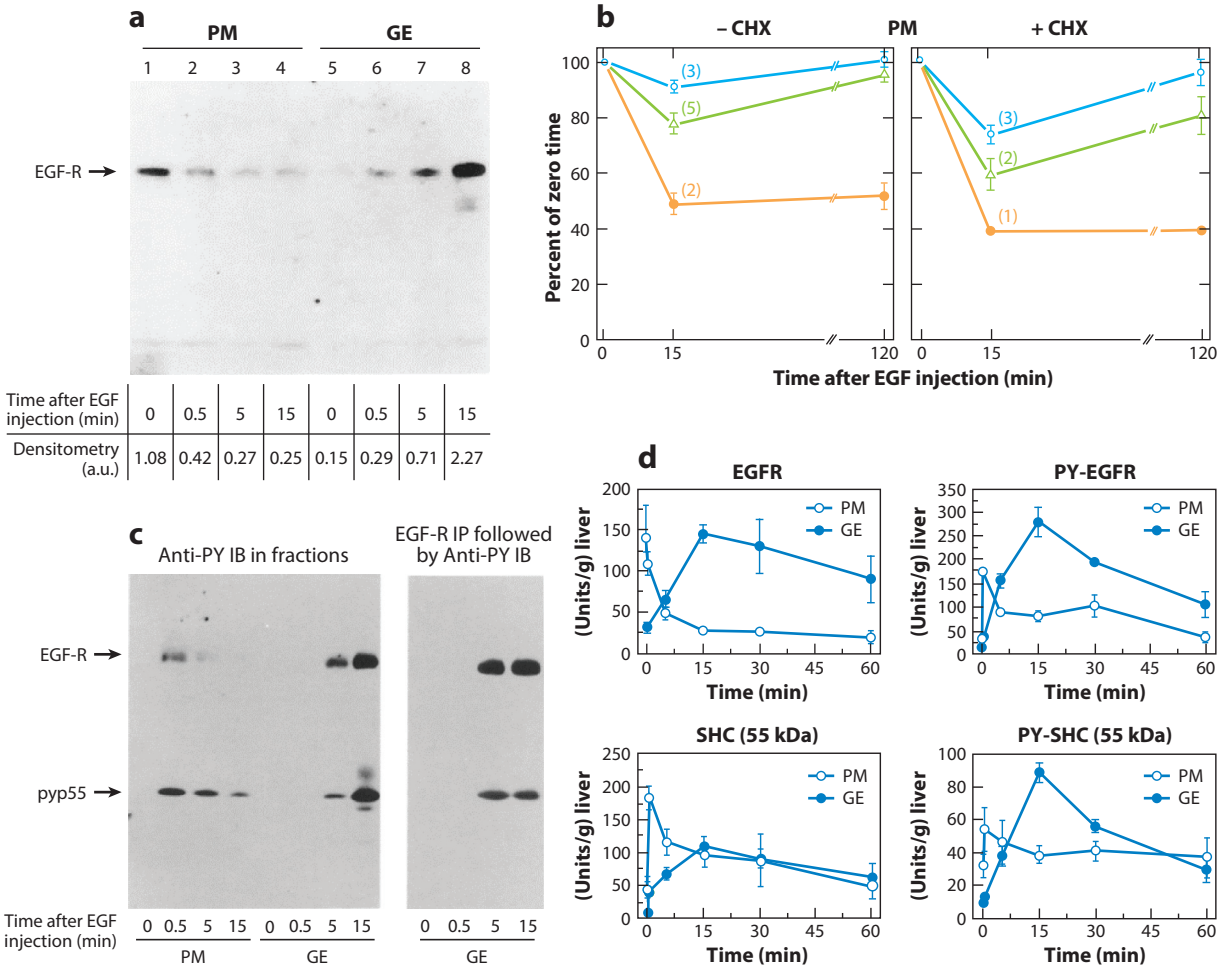

Figure 3

EGF–RTK internalization, recycling, and signaling in liver. (*a*) Immunoblot of EGF–RTK in plasma membrane (PM) and endosome (GE) fractions isolated from liver homogenates at the indicated times after administration of EGF. (*b*) Internalization of EGF–RTK at increasing subsaturating doses of EGF (*blue open circles*, 0.1 μg; *green open triangles*, 1 μg) with internalization but not recycling at saturating doses (*orange closed circles*, 10 μg). Recycling is not dependent on protein synthesis. Panels *a* and *b* adapted from Reference 25 with permission. ©1989 Lai et al. *J. Cell Bio.* 109:2741–50. doi: 10.1083/jcb.109.6.2741. (*c*) Tyrosine phosphorylation of EGF–RTK and associated SHC (pyp55) in plasma membrane and endosome fractions at various times after the administration of EGF to liver. Panel *c* adapted from Reference 26 with permission. ©1992 Wada et al. *J. Cell Bio.* 116:321–30. doi: 10.1083/jcb.116.2.321. (*d*) Estimation of EGF–RTK, PY-EGFR, SHC, and PY-SHC in plasma membrane and endosome liver fractions at various times after the administration of EGF. Panel *d* adapted from Reference 22 with permission. ©1994 Di Guglielmo et al. *EMBO J.* 13:4269–77. Abbreviations: CHX, cycloheximide; EGFR, epidermal growth factor receptor; IB, immunoblot; IP, immunoprecipitation; PY-EGFR, tyrosine phosphorylated EGF–RTK; PY-SHC, tyrosine phosphorylated SHC; SHC, Src homology 2–containing protein.

EGF–RTK dephosphorylation. This work confirms and extends the prior work done in liver parenchyma (32). Phosphotyrosine dephosphorylation of activated RTK is responsible for the termination of signal transduction. Remarkably, the endosomal membrane fusion machinery recruits tyrosine phosphatases to the cytosolic surface of signaling endosomes. In this way, the membrane fusion machinery regulates the different time courses of RTK activation and signaling

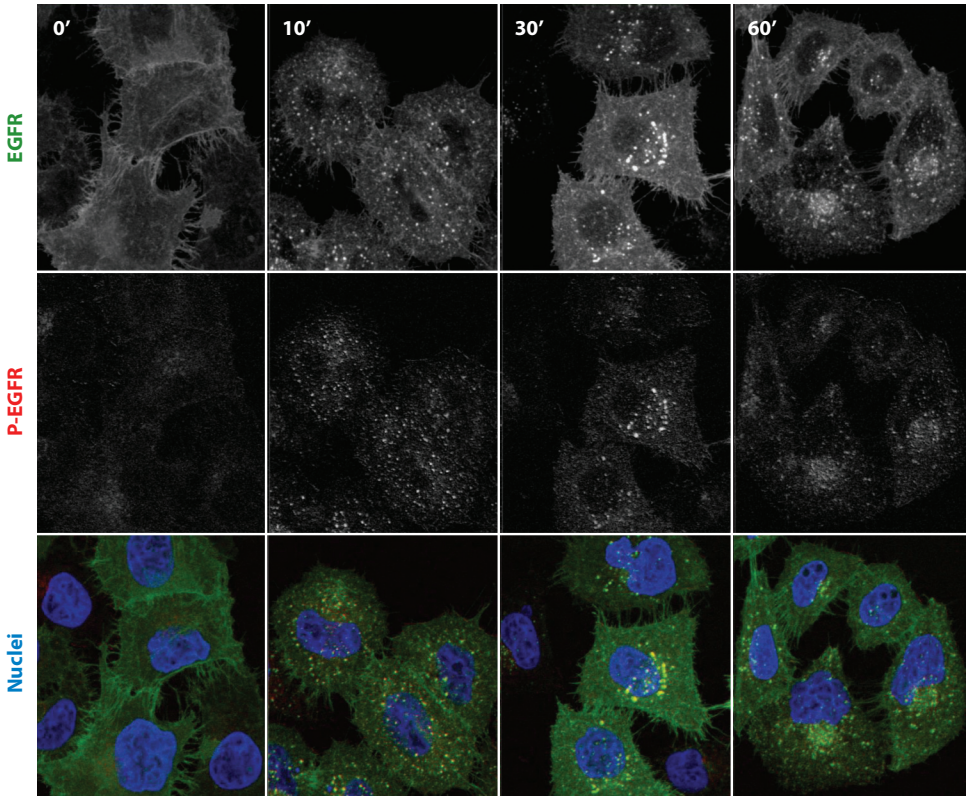

Figure 4

HeLa cell signaling endosomes. Visualization by FRET microscopy of the colocalization of EGF–RTK (EGFR) and tyrosine phosphorylated EGF–RTK (P-EGFR) in endosomes. HeLa cells transfected with a BAC transgene stably expressing EGFR–GFP under its endogenous promoter were incubated for the times indicated (0, 10, 30, or 60 min) with 10 ng/mL EGF. P-EGFR was visualized using mAb anti-phosphotyrosine directly labeled with Alexa Fluor 555. FRET signal for colocalization was detected with 458-nm excitation and 593-nm longpass spectral range using high-resolution confocal microscopy. Adapted from Reference 32 with permission. © 2015 Villaseñor et al. *eLife* 4:e06156. doi: 10.7554/eLife.06156. Abbreviations: BAC, bacterial artificial chromosome; EGF, epidermal growth factor; FRET, fluorescence (Förster) resonance energy transfer; GFP, green fluorescent protein; RTK, receptor tyrosine kinase.

for the EGF–RTK in HeLa cells, the nerve growth factor (NGF) receptor in PC12 cells, and the hepatocyte growth factor receptor MET (mesenchymal–epithelial transition factor) in hepatoblasts (32).

Signaling at the Plasma Membrane

In living cells, the dynamic relationship between monomeric and dimeric EGF–RTK has been quantified through quantum dot–based optical tracking of single molecules (33). The high-affinity state of EGF–RTK for binding EGF was the dimer, and the low-affinity state was the monomer. Phosphotyrosine modification of the RTK at position 1068 was shown within 1 min of EGF addition, and GRB2 recruitment to the cell membrane could be seen as rapidly as 14–46 s after EGF addition. Hence, ligand binding and signal transduction are seen at the cell surface in living

cells in culture with a time course of seconds. The RTK dimer is selected preferentially for ligand binding, leading to the RTK conformational changes that enable autophosphorylation and the recruitment of GRB2 at the cell surface (33).

GRB2: growth factor receptor–bound protein 2

SHC: Src homology 2–containing protein

RTK Internalization via Clathrin-Coated Vesicles

The endocytic rate constant (34) measures internalization as a function of the surface-occupied receptor with an observed endocytic rate constant (K_e) of occupied EGF–RTK >0.2 per min without a detectable lag (35). These recent estimates coincide with those made when the delineation of the endocytic rate constant was first defined (34, 36) as an apparent first-order rate constant for internalization. EGF–RTK internalization coincides with RTK autophosphorylation (35).

Recent studies show that >95% of total protein endocytic flux in mammalian cells occurs via clathrin-coated vesicles (37). Association of ligand-bound receptors with pre-existing clathrin-coated pits is one explanation for the absence of a lag in endocytosis (38). The adaptor protein 2 (AP-2) regulatory subunit of the clathrin coat associates with residues 973 and 977 at the C terminus of EGF–RTK, which are normally masked in the EGF–RTK dimer when unexposed to ligands (39). Upon ligand binding, this domain is exposed and associates with the clathrin heavy chain (LL motif at positions 1010 and 1011 and a tyrosine-based motif at position 974), assuring that internalization coincides with ligand binding to, and autophosphorylation of, the dimer (40). Furthermore, the phosphorylation of the Tyr-6 residue on the β subunit 2 of AP-2 by activated EGF–RTK promotes the association of LL motifs of the RTK with the clathrin-coated cage, thus concentrating activated EGF–RTK kinase in clathrin-coated pits (41).

For receptor internalization via clathrin-coated pits, actin and the membrane-scission protein dynamin are recruited 20–30 s prior to fission and the generation of a free clathrin-coated vesicle (42). The total time for a clathrin-coated vesicle to generate its coat and become a free vesicle has been estimated as approximately 100 s (43). Aside from the clathrin coat and adaptins, more than 30 different endocytic proteins have been documented to be recruited to a clathrin-coated structure before scission (43).

A cytosolic mediator of activated EGF–RTK signaling is GRB2, which, via its association with dynamin (44), regulates EGF–RTK access to the endocytic machinery. Scission of the EGF–RTK–containing clathrin-coated vesicle is mediated by dynamin. Indeed, dynamin-conditional knockout mouse fibroblasts have exhibited enhanced EGF–RTK autophosphorylation, enhanced and prolonged EGF–RTK ubiquitinylation, and reduced EGF–RTK degradation (44). Selective enhancement of tyrosine phosphorylation of the EGF–RTK substrate, the p66 isoform of the adaptor protein SHC, as well as a sustained activation of the EGF–RTK downstream kinase Akt has also been observed in the Tamoxifen-inducible, dynamin-conditional knockout mouse fibroblasts (45). Therefore, signaling has been concluded to be at the plasma membrane with internalization to access attenuation and with degradation machineries to terminate signaling. However, as described by Villaseñor and colleagues (32), a likely explanation is that surface signaling may accommodate and replace the unattainable endosomal signaling and regulation in cells devoid of dynamin.

Macropinocytosis

Internalization of the platelet-derived growth factor receptor (PDGFR) into macropinosomes was shown in Ras-transformed fibroblasts (46). Ras-mediated macropinocytosis was prevented by inhibitors of phosphatidylinositol 3-kinase (PI3K). Macropinosomes accumulate the marker Rabankyrin-5 (Rab5) as well as activated PDGFRβ upon addition of platelet-derived growth

factor (PDGF). Entry of activated PDGFRβ into macropinosomes and endosomes [as deduced by colocalization with the early endosome markers early endosome antigen 1 (EEA1) and Rab5] has led to the activation of the phospholipase Cγ (PLCγ)/protein kinase C (PKC) and PI3K/protein kinase B (Akt) pathways and anchorage-independent cell growth.

Endophilin-Mediated Endocytosis

The distinction between clathrin-dependent and clathrin-independent vesicular entry of EGF–RTK has been addressed by McMahon and colleagues (47, 48). Using a variety of cell lines, and especially human dermal primary fibroblasts, they have shown a rapid (within-seconds) association of EGF–RTK in surface tubulovesicular structures upon addition of EGF. These structures, distinct from clathrin-coated vesicles, colocalized with endophilin. These endophilin-marked structures required dynamin as well as endophilin for membrane scission from the cell surface. The mobile vesicles then rapidly fused with tubulovesicular endosomes to accumulate EGF–RTK.

Endosome Fusion and the Regulation of Signaling

A key regulatory event in endocytosis is early endosome fusion and fission (49, 50). Early endosomes are recognized through their markers, Rab5 and the Rab5 effector EEA1. After the addition of EGF, RTK signaling in endosomes activates p38 mitogen-activated protein kinase (MAPK) that in turn enhances the recruitment of EEA1 and the Rab4/5 effector membrane fusion protein, Rabenosyn-5, to early endosomes (51, 52). In this way, early endosome membrane fusion is regulated. This regulation generates signaling endosomes in which RTK autophosphorylation and RTK tyrosine phosphorylation of SHC occur (32). An attenuation mechanism thus balances signaling with RTK inactivation. Endosome-recruited phosphotyrosine phosphatases dephosphorylate the activated EGF–RTK. The protein tyrosine phosphatase, nonreceptor/Src homology 2 (SH2) (PTPN11/SHP2) domain has been identified as the relevant phosphotyrosine phosphatase based on RNAi screens affecting endosomal signaling (32). This same phosphotyrosine phosphatase also regulates EGF signaling in *Drosophila melanogaster* embryos. In this instance, signal transduction from the activated EGF–RTK is under the regulation of Corkscrew, the *D. melanogaster* ortholog of PTPN11/SHP2 (53).

Protein tyrosine phosphatase 1B (PTP1B) knockdown did not affect endosomal signaling (32), an effect that was previously predicted from a cell-free assay for endosomal phosphotyrosine phosphatase activity. This cell-free assay used isolated endosomes harboring tyrosine-phosphorylated activated RTK. An ATP-dependent dephosphorylation of the activated RTK that was unaffected in endosomes isolated from PTP1B knockout mice was observed (54).

As regulated by EEA1, Rabenosyn-5, VPS45 (the vacuolar protein sorting 45 homolog), and syntaxins 6 and 13, early endosome fusion in cells generates larger endosomes. These endosomes recruit PTPN11 and perhaps other phosphotyrosine phosphatases (PTP4A1, PTPN9, PTPN18, PTPRK) (32). This is rapidly followed by membrane fission to regenerate signaling endosomes retaining activated EGF–RTK for continued signaling. The dephosphorylated EGF–RTK is segregated into late endosomes for continued maturation and conversion to Rab7 endosomes. Ubiquitinylation by c-Cbl of the inactivated EGF–RTK is suggested to take place in late endosomes (32).

The extracellular concentration of EGF determines the proportion of EGF–RTK that is driven to the receptor degradation pathway (downregulation) and the proportion that accumulates in signaling endosomes. The number of these signaling endosomes augments with increased doses of EGF such that the mean phosphorylated EGF–RTK content per signaling endosome remains

EEA1: early endosome antigen 1

PTPN11/SHP2: protein tyrosine phosphatase, nonreceptor/Src homology 2 (SH2) domain that contains non-transmembrane protein tyrosine phosphatase

PTP1B: protein tyrosine phosphatase 1B

c-Cbl: c-Casitas B-lineage lymphoma proto-oncogene, E3 ubiquitin protein ligase

constant regardless of the dose of extracellularly administered EGF (32). In an imaging study by Villaseñor et al. (32), the signaling endosomes revealed EGF–RTK autophosphorylation as well as the recruitment and tyrosine phosphorylation of SHC with a similar time course as seen in experiments in liver parenchyma using subcellular fractionation (22). Signaling endosomes were also concluded to sustain extracellular signal–regulated kinase (ERK) activation and the stabilization of c-Fos (32).

PTEN: phosphatase and tensin homolog

PI(3)P: phosphatidylinositol 3-phosphate

Akt: protein kinase B

ERK: extracellular signal–regulated kinase

Dephosphorylated EGF–RTK accumulates in the late endosomes. As more extracellular EGF is added, an increase in density of inactivated EGF–RTK in the late endosomes can be found, but not an increase in their number. Therefore, signaling endosomes increase in number as extracellular EGF is increased, but late endosomes do not. At very high levels of extracellular EGF (>20 ng/mL), signaling endosomes have increased in number to their maximal extent. Adding even more EGF leads to an increased density of EGF–RTK in the signaling endosome, indicating that there is an upper limit to the generation of the number of signaling endosomes (32).

A similar paradigm is operative for EGF–RTK kinase activation and lipid signal regulation via the lipid phosphatase and tensin homolog (PTEN) (55). Imaging by confocal microscopy has colocalized endogenous PTEN in human embryonic kidney (HEK) 293 cells and mouse embryo fibroblasts (MEFs) to transferrin- and phosphatidylinositol 3-phosphate [PI(3)P]–containing endosomes (**Figure 5**). The calcium-binding region 3 loop within the C2 domain of PTEN (where known cancer-causing mutations are found) was shown to bind endosomal PI(3)P. PTEN acted on its substrate $PI(3,4,5)P_3$ in the endosome, where the EGF-mediated activation of PI3K to generate PI(3)P was shown and the diminishment of phosphorylated Akt. Hence PI(3)P, $PI(3,4,5)P_3$, and PTEN all colocalize to the EEA1 vesicle, which is shown to be the signaling endosome (32). Taken together, these studies establish a spatiotemporal regulation of signaling by the endosomal activated EGF–RTK kinase with attenuation through endosomal recruitment of protein tyrosine phosphatases such as PTPN11 and the lipid phosphatase PTEN.

EGF Signaling and Proliferation

Monitoring of signaling from the activated EGF–RTK to phosphorylated ERK using a reporter for ERK activity (Fra-1 in live cells, and c-Fos, c-Myc, and Fra-1 in fixed cells) revealed an unexpected phenomenon (56). Continuous incubations of cells with EGF revealed pulses of ERK activation whose frequency increased with EGF concentration. The frequency and duration of ERK pulses regulated entry into S phase as a consequence of sustained ERK activity, but the rate of entry into S phase was nonlinear with the output of the ERK pathway. This frequency modulation of signaling may be viewed as a reflection of analog to digital signal transduction by the signaling endosome characterized by Villaseñor et al. (32). The dose dependency of EGF to generate increasing numbers of phosphorylated EGF–RTK signaling endosomes each containing the same number of activated EGF–RTKs may explain the quantal nature of signaling observed by Albeck et al. (56). In this study, an enhanced pulse frequency of ERK activation was observed with increased doses of EGF. The proliferation rate was especially sensitive to low doses of EGF. At high EGF doses, the proliferation rate plateaued despite increased ERK activation. This finding may reflect the increase in the number of signaling endosomes at low doses of EGF. At high doses, the signaling endosome number no longer increased, but the phosphorylated EGF–RTK density increased in each signaling endosome and a greater proportion of inactivated RTKs targeted to the late endosome (32). The studies of Albeck et al. (56) reported the averaged signaling in individual cells, whereas Villaseñor et al. (32) measured signaling in individual endosomes. Thus, although the similarities between the two studies are striking, a direct comparison of the studies is purely speculative.

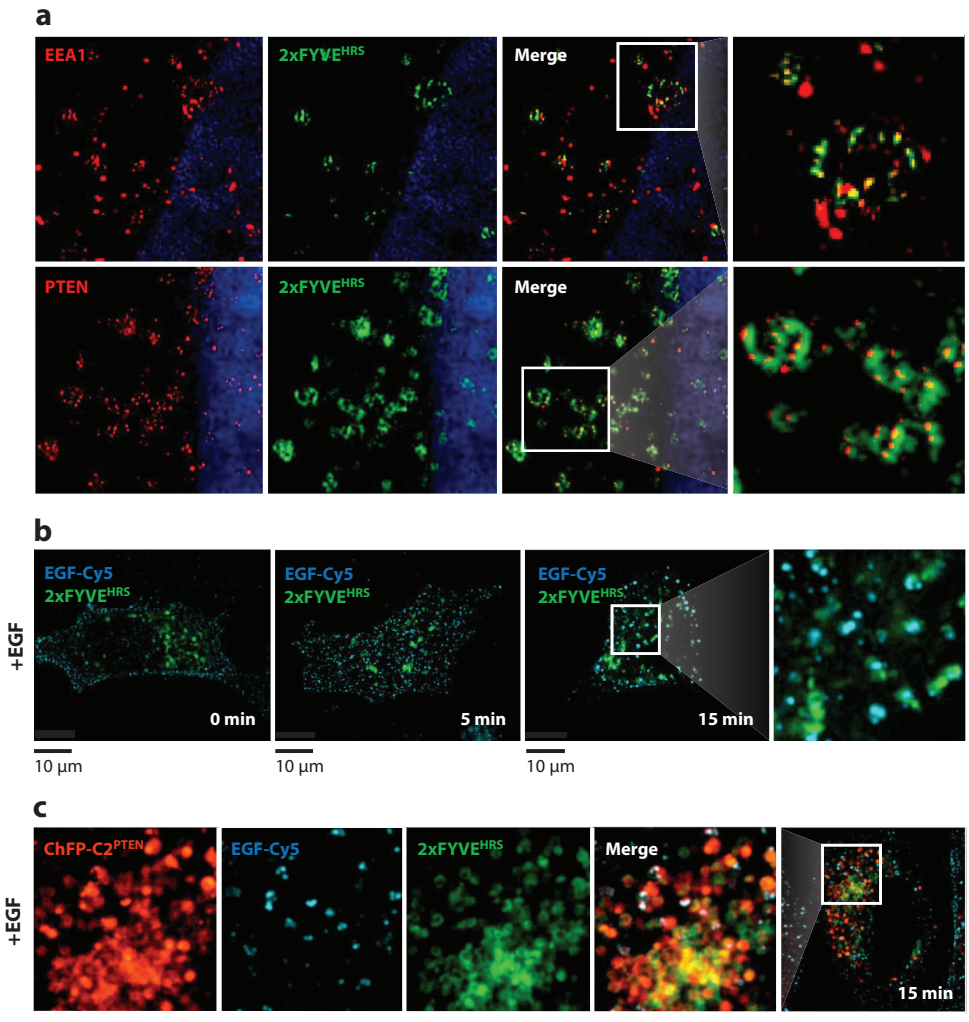

Figure 5

Localization of PTEN and EGF in endosomes. (*a*) Colocalization in HeLa cells of early endosome marker EEA1 and PTEN (visualized using a specific primary antibody and Alexa Fluor–labeled secondary antibodies) with GFP-tagged FYVE domain of HRS, a probe for PI(3)P. (*b*) Colocalization of EGF-Cy5 with GFP-tagged FYVE domain of HRS, a probe for PI(3)P, after 0-, 5-, and 15-min incubation with EGF-Cy5. (*c*) Colocalization of the ChFP-tagged C2 domain of PTEN as a marker for PI(3)P with EGF-Cy5 after 15-min incubation with EGF-Cy5 indicated a likely colocalization of EGF/EGF–RTK, PI(3)P, and PI(3,4,5)P_3 in the endosomes. Reprinted from Reference 55 with permission from Elsevier. Abbreviations: ChFP, mCherry fluorescent protein; EEA1, early endosome antigen 1; EGF, epidermal growth factor; GFP, green fluorescent protein; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; PI(3)P, phosphatidylinositol 3-phosphate; PTEN, phosphatase and tensin homolog.

ER-Mediated Fission Sorting and Maturation of Endosomes

Recent studies have implicated the endoplasmic reticulum in the sorting and fission events during endosomal maturation. Direct contact of the ER coincides with the fission of early and late endosomes harboring internalized EGF–RTK (57, 58). This model is supported by the discovery

of protrudin, the PI(3)P binding protein of the ER protein. Protrudin and Rab7 on the late endosome cooperate to generate contact sites between PI(3)P-containing late endosomes and the ER for sorting, segregation, and maturation of late endosomes (59).

The involvement of the ER-restricted PTP1B in receptor dephosphorylation (57, 60, 61) provides an additional assurance of receptor dephosphorylation after the prior dephosphorylating activities of endosomal PTPN11 and other phosphotyrosine phosphatases (32).

HRS: hepatocyte growth factor–regulated tyrosine kinase substrate

ESCRT: endosomal sorting complexes required for transport

Receptor Recycling

Recycling of plasma membrane constituents is extensive (62, 63). Subcellular fractionation of liver parenchyma after the administration of EGF revealed rapid endosomal accumulation of EGF–RTK, and recycling to the plasma membrane was revealed at low doses of EGF (**Figure 3*b***). However, a saturating dose of EGF led to RTK downregulation (25). As summarized in **Figure 1**, the generation of the recycling endosome is also likely mediated by an ER-regulated endosomal fission process at ER/endosome contact sites marked by the protein family with sequence similarity 21 (FAM21), a subunit of the actin nucleation WASH complex (58).

Ligand Degradation

Hepatic endosomes were shown to be a key locus of insulin degradation in vivo (30, 31). Cell-free assays with liver endosomes showed the ATP-dependent receptor dissociation and the degradation of both insulin (27, 64) and EGF (28). Cathepsin D, first described by Diment & Stahl (65, 66) in endosomes, was shown to be the major endosomal proteinase for insulin following its dissociation of insulin from the insulin receptor (29). Cathepsin B has been found to be responsible for cleavage of endosomal EGF (28).

The Late Endosome and Multivesicular Endosomes

The work of Villaseñor et al. (32) showed that the activated EGF–RTK substrate, HRS (hepatocyte growth factor–regulated tyrosine kinase substrate), likely associates with the larger late endosome segregated from the smaller EEA1 signaling endosome. HRS appears to play a role in sequestering dephosphorylated receptors to late endosomes. Although knockdown of HRS increased phosphorylated EGF–RTK, the activation of the transcription factor Elk-1 was not affected (67). This finding could be explained by the scaffolding-mediated association of HRS with the already segregated dephosphorylated EGF–RTK in the larger late endosome. HRS is a constituent of the ESCRT complex needed for the formation of multivesicular endosomes. However, interfering with the expression of other ESCRT proteins needed for the intralumenal budding and generation of multivesicular endosomes did not affect Elk-1 activation (67). Taken together, the studies of Villaseñor et al. (32) and Brankatschk et al. (67) indicate that EGF–RTK signaling to activate Elk-1–mediated transcription is unlikely to occur from late endosomes.

The EGF Signaling and Attenuation Interactomes

The characterization of proteins associating with activated EGF–RTK or insulin–RTK has been attempted by quantitative tandem mass spectrometry. In an early study, HeLa cells were incubated with EGF at 150 ng/mL for 10 min. The GRB2 Src homology 2 domain linked to Sepharose beads was used to isolate proteins in HeLa cell lysates. Six signaling proteins were characterized: EGF–RTK; SHC; SOS, the guanine nucleotide exchange factor; four subunits of

the clathrin-associated adaptin 2 protein complex; Casitas B-lineage lymphoma proto-oncogene B, E3 ubiquitin protein ligase (CblB), the ubiquitin conjugating enzyme; and PTPN11/SHP2, the phosphotyrosine phosphatase (68). PTPN11/SHP2 is known to be required for the regulation of EGF–RTK signaling (69) and the segregation of dephosphorylated EGF–RTK into the late endosome (32).

A similar strategy was used to study HeLa cells incubated for different times with saturating doses of EGF (70). Immunoprecipitation of endogenous EGF–RTK or phosphotyrosine proteins revealed a time course of tyrosine phosphorylation of EGF–RTK at six sites by 4 min after EGF stimulation. Tyrosine phosphorylation was maintained at 50% of maximal levels at 14 min after the addition of EGF to the HeLa cells. Aside from GRB2 and SHC, the transcription factor STAT5 was also characterized as associated with activated EGF–RTK.

Sousa et al. (45) expressed FLAG-tagged GRB2 in HEK 293 cells and, at various times after adding high levels of EGF, quantified GRB2-associated proteins by selected reaction monitoring. Monitoring of EGF–RTK, PTPN11/SHP2, PTN12, SHC, and VAV3 revealed peak accumulations 1–3 min after EGF administration. These studies showed a much more limited time course of EGF–RTK activation and signaling than those seen in the imaging studies described above (e.g., 32). This difference may be a consequence of restricted availability of the FLAG epitope for GRB network characterization. Availability of the FLAG epitope is the basis for the protein characterizations and, as the complexes build up, FLAG availability may become limited—especially at peak times of internalization into endosomes.

In Rat2 fibroblasts (71), FLAG-tagged SHC was maximally tyrosine phosphorylated after 2 min of EGF incubation. The time for maximal association with GRB2, EGF–RTK, SOS, c-Cbl, and the phosphotyrosine phosphatase PTPN11/SHP2 was also 2 min. PTPN11/SHP2 was characterized for EGF–RTK signaling by the Mann group (68) and deduced by Villaseñor et al. (32) as the endosomal phosphotyrosine phosphatase of EGF–RTK. In a study by Zheng et al. (71), 2 min was also the time point for maximal tyrosine phosphorylation for three sites characterized for EGF–RTK and two tyrosine phosphorylation sites on SHC. By comparing wild-type MEFs and GRB2-deleted MEFs, the associations were all found to be GRB2 dependent. For the Rat2 fibroblasts, by 10 min after incubation with EGF, SHC maintained a high level of tyrosine phosphorylation at PY239/240 as well as an association with GRB2, although SOS association was greatly diminished. Although a comparison of studies using protein localization (32) and mass spectrometry of protein complexes have found overall agreement with the protein interactomes of EGF–RTK and signaling scaffolds, the time courses are different. For the associated protein experiments, quantification is based on epitope availability for the immunoprecipitates or pull-downs, whereas for cell imaging all compartments are considered but still subject to the caveat of tag fidelity or epitope availability for localizations with antibodies.

Membrane-trafficking proteins have been characterized by quantitative mass spectrometry of protein complexes. In HEK 293 cells expressing EGF–RTK, EGF addition at 10 ng/mL resulted in the association of 5 subunits of the clathrin adaptor protein 2 (AP2) with tagged EGF–RTK 15 min after incubation of cells with EGF (72). Under these conditions, the retromer constituent sorting nexin 2 involved in endosomal protein traffic and the GTPase ADP-ribosylation factor 6 (ARF6) are associated. ARF6 recruitment to EGF–RTK in breast cancer cells is under the regulation of the tyrosine-phosphorylated p66 SHC isoform (73).

Mass spectrometry has also characterized ubiquitin-modified proteins after the addition of EGF to cells. One study characterized 265 different proteins that were mono- or polyubiquitinylated (74), using B82L fibroblasts or HeLa cells expressing human EGF–RTK and FLAG-tagged ubiquitin. The study assessed ubiquitinylated proteins after incubation with EGF (100 ng/mL) for 10 min at 37°C. Analysis of immunoprecipitates from antimonoubiquitin- or

antipolyubiquitin-specific monoclonal antibodies characterized ten different E3 ligases, of which three (c-Cbl, CblB, and Huwe1) are known to be tyrosine phosphorylated by activated EGF–RTK. This study also characterized three E2 enzymes and six deubiquitinylating enzymes. The ubiquitinylated β1 subunit of clathrin-adaptin protein 2 was characterized as well as the clathrin-coated pit protein EPS15, required to recruit EGF–RTK to clathrin-coated pits (75). Other ubiquitinylated proteins included EPS15L1 as well as the Rab5 guanine nucleotide exchange factor, Rabex 5 (76), which is also a ubiquitin E3 conjugating enzyme.

CblB: Cbl proto-oncogene B, E3 ubiquitin protein ligase

IRS1 and IRS2: insulin receptor substrates 1 and 2

GAB1: GRB2-associated binding protein 1

When HEK 293 T cells were treated with orthovanadate to inhibit phosphotyrosine phosphatase activity (77), quantitative mass spectrometry was used to compare c-Cbl interactions in cells containing c-Cbl with c-Cbl knockdown cells. The CD2-associated protein (a c-Cbl–interacting protein of 85 kDa), GRB2, and sortin nexin 18 were characterized (77).

In summary, imaging experiments and those employing quantitative mass spectrometry of protein complexes point to the location of signaling and attenuation complexes (**Figure 1**). Although signaling and attenuation proteins may be seen at the surface, such as in endophilin knockdown or dynamin knockout cells or in cells incubated with EGF at 4°C (72), these findings do not represent the situation under physiological conditions at 37°C at which, within the endosomal apparatus, EGF–RTK signaling, sorting, and inactivation transpires.

Insulin Signaling

Balbis et al. (78) studied insulin receptor substrates 1 and 2 (IRS1 and IRS2) as well as PI3K and Akt in isolated hepatic endosomes and plasma membranes. Following insulin administration, the levels of IRS1 and IRS2 decreased in plasma membranes and increased by 100–150% in endosomes. PI3K rapidly increased in both plasma membranes and endosomes and the bulk of PI3K was associated with IRS1 and IRS2. Akt was also rapidly recruited to both compartments but achieved higher specific enzyme activity in endosomes than in plasma membranes. These observations support a key role for endosomes in insulin signaling.

Studies in mice using homologous recombination with null alleles for insulin–RTK, IRS1, or IRS2 alone or in combination have revealed the key roles of IRS2 in insulin signaling in liver (79). Mice deleted for liver-specific class II PI3K isoform γ (PI3K-C2γ) showed reduced insulin sensitivity and lower glycogen levels in liver (80) as well as reduced Akt2 phosphorylation after insulin stimulation of hepatocytes. Insulin administration to mice showed that at 15 min, $PI(3,4)P_2$ in Rab5-positive endosomes was reduced fourfold in the $PI(3,4)P_2$ liver content of mice deleted for liver PI3K-C2γ. In HEK 293 cells, COS cells, and primary hepatocytes expressing Myc-tagged PI3K-C2γ, insulin stimulation led to colocalization of PI3K-C2γ with Rab5 vesicles and therefore early endosomes. Insulin-dependent activation of Akt2 in early endosomes was shown to be dependent on PI3K-C2γ. In this way, the liver endosome through PI3K-C2γ regulates glucose metabolism.

Several recent studies have used tandem mass spectrometry to deduce constituents of the insulin-signaling network. Immune-precipitates of IRS1 from homogenates of muscle from biopsies of glucose-tolerant lean, glucose-tolerant obese, and type 2 diabetic patients revealed 122 IRS-1 interacting proteins (81). Of these, PI3K α and β subunits showed an insulin-dependent increased association with IRS1 in muscle of lean but not that of the obese or type 2 diabetic subjects.

3T3-L1 adipocytes have been characterized by quantitative mass spectrometry for insulin signaling after incubation with 150 nM insulin (82). The results at three time points (5, 15, 45 min) compared with untreated cells showed a major increase (>10-fold) in tyrosine phosphorylated sites on the insulin–RTK, its substrate IRS1, and the GRB2-associated binding protein 1 (GAB1),

including the sites in GAB1 that recruit PTPN11/SHP2 as well as six other proteins. Tyrosine phosphorylation of the insulin receptor and IRS1 was maintained for 15 min, whereas GAB1 progressively decreased in phosphotyrosine content. IRS2 displayed a >3-fold increase in tyrosine phosphorylation by 5 min, and PI3K and PTPN11/SHP2 showed a 30% increase by 5 min that increased further by 15 and 45 min.

Kruger et al. (83) assessed differentiated brown adipocytes for insulin-dependent (100 nM) tyrosine phosphorylation at 0, 1, 5, 10, and 20 min of incubation. A 20-fold increase of tyrosine phosphorylation of insulin–RTK was found at 1–5 min and remained at 85% maximal by 20 min; a similar pattern was found for IRS1 (but not IRS2) that peaked at 1 min and declined thereafter. The downstream regulatory and catalytic subunits of PI3K were maximally phosphorylated at 1–5 min and declined thereafter, whereas PTPN11/SHP2 phosphorylation increased at 1 min and was maintained for 15 to 20 min.

A recent study in mice showed that the silencing of expression of the GTPase Rab5, the marker of early endosomes, resulted in a marked reduction in hepatic endosomes (84). A corresponding inhibition of gluconeogenesis and glycogen breakdown was explained by a signal transduction pathway from the endosome that enabled the transcription factor cAMP response element binding protein (CREB) to be activated and its downstream transcription factors, PGC1α and ChREBP, to be expressed fully. In the Rab5 knockdown mice, PGC1α and ChREBP levels were significantly reduced. The Forkhead box protein O1 transcription factor was highly phosphorylated and failed to translocate to the nucleus, thus explaining the defects in gluconeogenesis and glycogen breakdown. Furthermore, in a mouse model for type 2 diabetes with a missing leptin receptor ($Lepr^{db/db}$ mice), the Rab5 knockdown in liver fully reversed the hyperglycemia in these mice. This finding corresponded to the marked reduction in glucose-6 phosphatase and phosphoenoyl pyruvate carboxy kinase 1 (PCK1/PEPCK1) expression as also observed in livers of control normal mice after Rab5 knockdown (84).

Taken together, these studies reveal distinct signaling proteins for insulin–RTK as compared with the proteins seen after EGF–RTK activation. For both insulin–RTKs and EGF–RTKs, however, PTPN11/SHP2 is observed as the relevant protein phosphotyrosine phosphatase.

Extracellular Vesicles

The fusion of multivesicular endosomes with the plasma membrane leads to the delivery of the intralumenal vesicles to the cell exterior as exosomes (85). Such vesicles have been suggested to transfer mutant oncogenic EGF–RTK from tumor cells (86) and for autocrine Wnt–planar cell polarity signaling in breast cancer cell migration (87). However, extracellular vesicles originate not only from the multivesicular endosome but also by budding and fission from the plasma membrane itself (88). The regulation of multivesicular endosome fusion with lysosomes or the plasma membrane is under active study (87, 89). Nevertheless, the role of extracellular vesicles in transferring oncogenic and metastatic potential is well documented, and the sorting out of the trafficking mechanisms that regulate such vesicular-mediated intercellular communication is under detailed study (e.g., 90).

Maturation of Multivesicular Endosomes to Lysosomes

The degradation of internalized EGF–RTK takes place in the lysosome (91). Repeated fusions of endosomes with lysosomes generate endolysosomes with degradation of the intralumenal vesicles harboring internalized receptors (28, 92–94). The generation of multivesicular endosomes has been implicated in signal transmission, especially for Wnt signaling. At the step of intravesiculation

SIGNALING AND AUTOPHAGOSOMES

Nutrient sensing through TORC1 kinase negatively regulates the formation of autophagosomes. During starvation, TORC1 dissociates from UNC-51-like kinase 1 (ULK1), thereby activating the latter to initiate autophagosome formation from preautophagosomal structures. Autophagosomes are generated in proximity to the endoplasmic reticulum and involve the ULK1-mediated activation of PI3K (146). Autophagy is also regulated by activated AMPK (AMP-dependent protein kinase). It is presently uncertain how the EGF and insulin-signaling endosomes use membrane trafficking to create a response to nutrient sensing.

of vesicles through the ESCRT machinery recruited to late endosomes, the segregation of glycogen synthase kinase 3β (GSK-3β) and axin into the lumen of the internal vesicles of the multivesicular endosome leads to their removal from the cytosol. This sequestration event enables the activation of Wnt signaling for growth and differentiation (95–97). GSK-3β also plays a role in controlling the flux of endocytosis through the clathrin-coated vesicles. Consequent to EGF-induced activation of Akt, GSK-3β is phosphorylated on the cytosolic surface of endosomes. The authors concluded that this phosphorylation regulates the selection of dynamin isoforms that affect the flux of clathrin-mediated endocytosis and endosomal signaling (98).

The balance between receptor degradation (downregulation) and recycling in EGF–RTK and insulin–RTK is dependent on the dose of EGF or insulin administered to cells (25, 32, 99–101). The endolysosome/lysosome locus is itself a sensor for amino acids generated from intralysosomal protein degradation. At this locus, cell growth is regulated through the target of rapamycin (TOR) kinases, and through these kinases protein translation is regulated through cap-dependent initiation factors (see the sidebar, Signaling and Autophagosomes). This has been illustrated recently by a pathway from the lysosomal amino acid transporter SLC38A9 that allows arginine to access the cytosol and associate with the GTPase RAG and allows Ragulator to regulate TOR complex 1 (TORC1) activation (102). Thus, through its signaling system of RAGs, the Ragulator v-ATPase GATOR (GAP activity toward RAGs), and folliculin complexes, the lysosome enables amino acid availability through lysosomal degradation of proteins and activation of TORC1 (103). A role for vacuolar acidification in EGF signaling at the endosome to mammalian TOR complex 1 (mTORC1) has also been uncovered. In this instance, EGF administration led to the recruitment of Ras Homolog Enriched In Brain to endosomes, lysosomes, and TORC1 activation (104). Furthermore, the EGF–RTK substrate p66 SHC regulates glycolysis through mTOR as an antagonist of insulin and mTOR signaling (105).

As illustrated by studies of the insulin–RTK and EGF–RTK, membrane trafficking from the plasma membrane to the lysosome coordinates the RTK-mediated sensing of the extracellular environment through the levels of extracellular insulin and EGF. In this way, cell metabolism, cell growth, and cell division are regulated.

SERINE/THREONINE KINASE RECEPTORS

The importance of endosomal signaling is well established for the transforming growth factor β (TGF-β) receptor. The signal transduction pathway initiated by cell surface TGF-β receptors is dependent on ligand binding as well as receptor internalization and trafficking (106–111). The two internalization pathways used by TGF-β receptors are clathrin dependent and caveolae/membrane raft dependent, which promote TGF-β receptor signaling (106–111) and degradation (112–114), respectively.

TORC1: target of rapamycin complex 1

The canonical TGF-β receptor signaling pathway is facilitated by the SMA-Mothers Against Decapentaplegic (SMAD) anchor for receptor activation protein (SARA), which binds the receptors and recruits SMAD2/3 to the membrane of early endosomes (111). TGF-β signaling is antagonized by the inhibitory SMAD7. SMAD7 interacts with the TGF-β type I receptor in membrane rafts/caveolae and recruits the E3 ligases SMAD ubiquitin regulatory factors 1 and 2 (SMURF1 and SMURF2) to direct ubiquitin-dependent degradation of the TGF-β receptor (115–117) and dephosphorylation of R-SMAD proteins by a nuclear resident small phosphatase (118, 119). Although cell surface TGF-β receptors reside in both membrane raft and nonraft membrane domains (106, 112, 113, 120), the determinant(s) that controls partitioning into these two membrane domains is unclear.

TGF-β: transforming growth factor β

AP2: adaptor protein 2

GPCR: G protein–coupled receptor

A short peptide sequence ($I_{218}I_{219}L_{220}$) on the cytoplasmic domain of TGF-β type II receptor is the major signal for clathrin-mediated endocytosis (121), and TGF-β receptors associate with both clathrin and AP2 (122). However, the cytosolic TGF-β type II receptor domain also contains consensus sequences that were previously identified as caveolin scaffolding domains (123), and the TGF-β type I receptor, which is the major binding partner of the TGF-β type II receptor, has also been shown to associate with Caveolin-1 (113, 124).

Recently He and colleagues (125) have shown that both pathways can converge during TGF-β receptor endocytic trafficking. Using total internal reflection fluorescence microscopy, they demonstrated that clathrin-coated vesicles and caveolar vesicles could fuse underneath the plasma membrane to form vesicles containing both clathrin and Caveolin-1. Furthermore, the delivery of these vesicles to EEA1/Caveolin-1–positive endosomes was shown to be dependent on Rab5 activity. Remarkably, the proteins that regulate TGF-β signaling and degradation were found in different regions of the double-positive vesicles. These results are predicted by the model proposed by Zerial & McBride that describes different members of the Rab family occupying common endocytic vesicles in a mosaic pattern (126). Taking this model into account, proteins that propagate TGF-β signaling (e.g., SARA) would occupy the nonraft regions of the vesicles and retain receptors in those regions, whereas SMURF2/SMAD7 proteins, which target receptors for degradation, would occupy the raft regions and direct receptors toward the degradation pathway.

G PROTEIN–COUPLED RECEPTORS

The seven-transmembrane G protein–coupled receptor (GPCR) family comprises over 600 different receptors. Each responds to different ligands, ranging from chemical neurotransmitters, peptide hormones, lipids, and taste and odorant molecules to physical stimuli (e.g., light) (127). Activated GPCRs couple to inactive, guanosine diphosphate (GDP)–bound heterotrimeric G proteins ($G_{\alpha\beta\gamma}$) to form a transient ternary complex (128). This interaction decreases GDP binding to G_α subunits and the replacement with GTP, followed by G_α and $G_{\beta\gamma}$ dissociation. Both G_α-GTP and $G_{\beta\gamma}$ subunits can then interact with various membrane-bound proteins that initiate or suppress effector activities, which in turn, regulate second messengers, kinase cascades, or ions involved in a wide range of physiological activities (128).

Using biosensors, parathyroid hormone type 1 receptors have been shown to sustain cAMP production after internalization of ligand–receptor complexes (129–131). PTH stimulation induced sustained cAMP signaling, which correlated with internalized PTH–PTHR complexes accessing stimulatory G protein (G_S) and adenylate cyclases on early endosomal membranes. Similar results were observed with the thyroid-stimulating hormone receptor (132). Remarkably, these observations were not specific to stimulatory G proteins: Receptors activating inhibitory G protein (G_i) activity, such as the sphingolipid S1P receptor, exhibited sustained G_i-dependent signaling after internalization and trafficking to the *trans*-Golgi network (133). To date, modulation of cAMP

signaling mediated by internalized GPCRs has been reported for other peptide hormone receptors, such as the glucagon-like peptide 1 receptor (134), the pituitary adenylate cyclase–activating polypeptide type 1 receptor (135), and the vasopressin type 2 receptor (136).

Aside from accessing stimulatory or inhibitory G proteins on the endosomal membrane, extended GPCR signaling occurs post endocytosis via the scaffolding properties of β-arrestins, which allow internalized GPCR to activate ERK1 and ERK2 (137, 138). Furthermore, G_α subunits may regulate membrane trafficking through a role in addition to that of signaling. Interaction with growth and differentiation factor-associated serum protein-1 and dysbindin has been suggested to regulate the sorting of GPCRs into multivesicular endosomes via the ESCRT pathway (139). Hence, membrane trafficking appears to be a common regulator of signaling for RTKs, receptor serine/threonine kinases, and GPCRs.

MEMBRANE TRAFFICKING AND SIGNALING

The importance of membrane trafficking for RTK signal transduction has been long recognized (e.g., 140). RTK signaling has been demonstrated repeatedly in endosomes (e.g., 141), as has the localization of RTK signal transduction proteins in endosomes (e.g., 19; also see 142, 143). In this way, a target cell's response to insulin or EGF is mediated by its respective RTKs, whose rapid internalization via vesicles assures that signal transduction occurs at the right place and at the right time in the target cell. The endosomal location for signaling assures that signaling may be switched off by the recruitment of protein tyrosine phosphatases, such as PTPN11/SHP2, and other phosphatases, such as density-enhanced phosphatase-1 (144). Endosomal membrane fusion and traffic regulate the extent of signal transduction. Although large-scale screens have uncovered a large number of previously unrecognized membrane-trafficking constituents linked to signal transduction (e.g., 145), the complete elucidation of the biochemistry of RTK activation and intracellular signal transduction is the ultimate goal.

SUMMARY POINTS

1. Ligand-mediated receptor internalization of EGF and insulin concentrates activated RTKs in signaling endosomes.
2. Signaling is regulated by phosphotyrosine phosphatases recruited to the signaling endosome.
3. Membrane trafficking regulates signaling through the segregation of phosphotyrosine dephosphorylated receptors into late endosomes via membrane fusion and fission regulated by the endoplasmic reticulum.
4. Signaling is terminated via additional endosomal ligand degradation followed by receptor sequestration through intravesiculation into multivesicular endosomes and their fusion with lysosomes.
5. The paradigm of endosomal regulation of signaling has been found for serine/threonine kinase receptors, such as for TGF-β, as well as GPCRs.

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