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Compartmentalization of EGFR in Cellular Membranes: Role of Membrane Rafts

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ABSTRACT

There is now abundant evidence that the intracellular concentration of the EGFR and many other receptors for peptide hormones and growth factors is important for the temporal and spatial regulation of cell signaling. Spatial control is achieved by the selective compartmentalization of signaling components into endosomes. However further control may be effected by sequestration into sub-domains within a given organelle such as membrane rafts which are dynamic, nano scale structures rich in cholesterol and sphingolipids. Current data suggest the presence of EGFRs in non-caveolae membrane rafts. High doses of EGF seem to promote the sorting of EGFR to late endosomes through a raft/cholesterol dependant mechanism, implicating them in EGFR degradation. However our work and that of others has led us to propose a model in which membrane rafts in late endosomes sequester highly active EGFR leading to the recruitment and activation of MAPK in this compartment. J. Cell. Biochem. 109: 1103–1108, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: EGFR; COMPARTMENTALIZATION; MEMBRANE RAFTS; ENDOSOMES

The binding of Epidermal Growth Factor to its receptor (EGFR) is the initial step leading to the activation of cell signaling. Receptor occupancy is rapidly followed by internalization of EGFR into endosomes (ENs) [Lai et al., 1989a,b], originally regarded as a mechanism for receptor down regulation [Wells et al., 1990]. However, the concentration in ENs of highly tyrosine phosphorylated receptor kinases [Kay et al., 1986; Khan et al., 1986] and the augmented recruitment of key signaling modulators to the endosomal receptor argued for its role in cellular signaling [Di Guglielmo et al., 1994; Sorkin et al., 2000]. There is now abundant evidence that the intracellular concentration of the EGFR and many other receptors for peptide hormones and growth factors is important in the temporal and spatial regulation of cell signaling [Bevan et al., 1996; Sorkin and Von Zastrow, 2002; Teis and Huber, 2003; Miaczynska et al., 2004].

Spatial control is achieved by the selective compartmentalization of signaling components into endosomes [Bevan et al., 1996]. However, further control may be effected by sequestration into sub-domains within a given organelle such as membrane rafts which are dynamic, nanoscale structures rich in cholesterol and sphingolipids [Simons and Toomre, 2000; Hancock, 2006]. It has been proposed that the localization of EGFR and other receptors in rafts modulates their signaling properties [Pike, 2005; de Laurentiis et al., 2007]. In this review we discuss the role of membrane rafts in EGFR signaling with a focus on the role of endosomal membrane rafts in this process.

RAFTS IN CELLULAR MEMBRANES

It is now clear that membrane lipids are not randomly distributed in the cell but interact with proteins to form domains that segregate from the bulk of the membrane [Simons and Toomre, 2000; Hancock, 2006]. These domains, referred to as membrane rafts, were first postulated to explain the sorting of Golgi proteins to the apical plasma membrane (PM) of polarized MDCK epithelial cells [van Meer et al., 1987; Simons and van Meer, 1988]. More recent studies concentrated on establishing the existence of membrane rafts at the PM, although their presence in intracellular membranes is likely [Helms and Zurzolo, 2004].

Early studies used a combination of cold detergent extraction and gradient centrifugation to isolate putative rafts [Brown and Rose, 1992; Brown and London, 1998]. Thus, when cell lysates where treated with cold Triton X-100 followed by sucrose gradient

Abbreviations used: CCP, clathrin coated pits; CT-B, cholera toxin B; DRMs, detergent resistant membranes; EGFR, epidermal growth factor; EM, electron microscopy; ENs, endosomes; GM1, ganglioside GM1; GPI-AP, glycosyl phosphatidyl inositol anchored protein; MBCD, methyl beta cyclodextrin; PLAP, placental alkaline phosphatase; TfR, transferrin receptor.

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centrifugation, detergent resistant membranes (DRMs) were isolated as structures of low buoyant density. The enrichment in DRMs of cholesterol and sphingolipids was consistent with regarding DRMs as a cell fraction in which membrane rafts were isolated and concentrated [Brown and Rose, 1992; Brown and London, 1998]. However, this method of isolating membrane raft became a matter of controversy [Munro, 2003]. Although it is clear that DRMs do not represent intact rafts in vivo, it would appear that proteins co-purifying with DRMs are associated with hydrophobic environments in the membrane—very likely to be membrane rafts [Shogomori and Brown, 2003].

More recently, fluorescence resonance energy transfer (FRET), single particle tracking and electron microscopy were utilized to identify membrane rafts [Hancock, 2006]. These different imaging techniques have converged to demonstrate that in the resting state rafts are observable as highly dynamic, nanoscale lipid domains [Hancock, 2006] which can be clustered with antibodies or hormone stimulation into larger, more stable structures [Brown and London, 1998].

Caveolae are morphologically observable membrane invaginations, with a size range between 50 and 100 nm, enriched in caveolin oligomers [Anderson, 1998]. Some caveolae appear to constitute a subset of membrane rafts which have been implicated in the modulation of lipid trafficking, signal transduction, and endocytosis of viruses and toxins [Simons and Toomre, 2000; Parton and Richards, 2003; Zurzolo et al., 2003].

THE EGFR IS ASSOCIATED WITH MEMBRANE RAFTS

The EGFR is a tyrosine kinase receptor that can be activated by the binding of its cognate ligand [viz. EGF or TGF(α)]. The EGFR ligand complex forms homo or hetero dimers with other members of the Erb family resulting in EGFR autophosphorylation and activation with subsequent phosphorylation of downstream signaling proteins [Lai et al., 1989a; Jorissen et al., 2003]. The activated EGFR is rapidly internalized into early endosomes and subsequently recycled to the plasma membrane or sorted to late endosomes–lysosomes for degradation [Lai et al., 1989b].

Earlier work used a detergent free method [Smart et al., 1995] to show that the EGFR is highly concentrated in a low buoyant density caveolin-enriched fraction. This result was interpreted as the accumulation of EGFR in caveolae [Mineo et al., 1996; Furuchi and Anderson, 1998]. Subsequently it was shown that, at low doses of EGF, the EGFR internalized exclusively through a clathrin-dependent mechanism; whereas at high doses the EGFR internalized via clathrin-independent but raft-dependent process [Sigismund et al., 2005, 2008]. The authors also showed that the EGFR was recruited to caveolae only at high doses of EGF [Sigismund et al., 2005]. Both results appear to suggest that the EGFR could be internalized via caveolae at high doses of ligand.

This hypothesis was tested by Kazazic et al. who showed that a high dose of EGF did not increase the mobility of caveolae, or the recruitment of EGFR to caveolae, arguing against an involvement of caveolae in EGFR internalization and signaling [Kazazic et al., 2006, 105]. In agreement with these results we recently showed that a high dose of EGF induced rapid internalization of EGFR in DRMs isolated from liver PM without altering the level of caveolin in this DRM fraction [Wang et al., 2009]. Furthermore, the immuno-isolation of caveolin rich elements [Waugh et al., 1999] and immuno-EM [Ringerike et al., 2002; Puri et al., 2005] confirmed a lack of co-localization of caveolin with the EGFR. Finally Sigismund et al. [2008] recently showed that knocking down caveolin-1 with siRNA did not affect EGF-induced EGFR internalization, degradation, or signaling. Thus, the available data indicate that most EGFRs do not co-localize to caveolae; nor does the disruption of caveolae alter EGFR trafficking and signaling.

Do the available data support the presence of EGFR in non-caveolae membrane rafts? The high solubility of EGFR in Triton X-100 [Roepstorff et al., 2002; Pike et al., 2005] suggests that EGFRs do not reside in rafts or that they reside in a subclass of rafts with a lower dependence on cholesterol. A recent study by Hofman et al. [2008] appears to support the latter hypothesis. Their results suggested that the EGFR, but not the TfR, concentrated in lipid shells with GM1 and that this co-localization was independent of cholesterol. However, GPI-GFP co-localized with GM1 but not with the EGFR; and this interaction was dependent on cholesterol [Hofman et al., 2008]. Upon EGF stimulation, the GPI containing elements seemed to coalesce with the EGFR into a nanoscale raft platform, an interaction not dependent on cholesterol [Hofman et al., 2008]. These results suggest the existence of two classes of rafts in PM. The EGFR appears to be associated with the less ordered domain which might explain why EGFR is largely soluble in Triton-X100.

Further evidence for the presence of the EGFR in PM rafts comes from morphological studies by immuno-EM. Ringerike et al. [2002] showed that the EGFR, but not the TfR, co-localized with PLAP patched rafts at the PM of Hep-2 cells. This was extended by the demonstration that EGFR, but not TfR, localized to GM1-rich regions of the PM [Puri et al., 2005]. Both morphological analyses determined that 40–70% of EGFR in PM was located in raft domains [Ringerike et al., 2002; Puri et al., 2005]. This contrasts with our observation that of others that only around 6% of EGFR in purified rat liver PM can be isolated in DRMs [Puri et al., 2005; Balbis et al., 2007; Wang et al., 2009]. Taken together these data suggest that the EGFR may reside in at least two different raft compartments—one that is highly dependent on cholesterol (Triton X-100 insoluble) and the other, a more disordered domain that is less dependent on cholesterol.

EGFR SIGNALING AND MEMBRANE RAFTS

Most studies that have evaluated the involvement of membrane rafts in EGFR signaling relied on subcellular fractionation methods to isolate raft/caveolar enriched structures. Using a detergent-free method Mineo et al. [1996] showed that, in the basal state, EGFRs are enriched in raft/caveolar fractions isolated from Rat-1 cells. After EGF stimulation EGFRs disappeared from this raft-enriched fraction; and activated Raf-1 was recruited to this fraction corresponding to the activation of MAPK [Mineo et al., 1996; Furuchi and Anderson, 1998].

Studies, based on Triton X-100 extraction to prepare DRMs, suggested membrane rafts have a positive role on EGFR signaling

[Zhuang et al., 2002; Puri et al., 2005; Balbis et al., 2007; Wang et al., 2009]. Puri et al. [2005] observed that following EGF the EGFR, Shc and Grb2 were selectively recruited to a low buoyant density/Triton X-100 insoluble fraction isolated from HeLa cells. Zhuang et al. [2002] demonstrated that a small proportion of the pool of EGFR was recovered in a Triton X-100 insoluble fraction and became highly Tyr-phosphorylated in response to EGF compared with soluble EGFR. They also showed that EGFR and Akt phosphorylation was decreased when cholesterol was removed and restored when cholesterol was added [Zhuang et al., 2002]. In our studies we found that in response to EGF the EGFR in the DRMs became more highly Tyr-phosphorylated and more markedly associated with Shc and Grb2 compared to EGFRs in the original PM fraction [Balbis et al., 2007; Wang et al., 2009], indicating that EGFRs in DRMs become more highly activated than those in the non-raft PM.

Studies employing cholesterol depletion to effect raft dissolution have yielded conflicting results. Thus, though cholesterol depletion was found to inhibit EGF-induced recruitment of Raf-1 to a raft fraction [Furuchi and Anderson, 1998] MAPK activation and DNA synthesis were greatly augmented [Furuchi and Anderson, 1998] suggesting a negative role for membrane rafts in EGF signaling. Chen et al. showed that cholesterol depletion induced EGFR dimerization and Tyr-phosphorylation as well as MAPK activation [Chen and Resh, 2002]. Other studies, using cholesterol depletion in various cell lines, also concluded that EGFRs in rafts are inhibited in regard to EGF-activation and downstream signaling [Pike and Casey, 2002; Ringerike et al., 2002; Roepstorff et al., 2002]. These effects were attributed to cholesterol depletion since the repletion of cellular cholesterol had the opposite effect [Pike and Casey, 2002; Ringerike et al., 2002; Roepstorff et al., 2002]. Thus, it was proposed that full activation of the EGFR occurred only on exiting membrane rafts.

There is therefore an apparent contradiction with respect to the role of membrane rafts in EGFR signaling. However, the work assigning a negative role to membrane rafts on EGFR signaling is largely based on cholesterol extraction [Pike and Casey, 2002; Ringerike et al., 2002; Roepstorff et al., 2002]. These studies used concentrations of MBCD $>$ 5 mM under the assumption that MBCD disrupts only membrane raft domains. However, it was shown that these concentrations of MBCD disrupted clathrin coated pits [Rodal et al., 1999]; and that even at a concentration of MBCD greater than 2 mM primary rat hepatocytes became permeable to antibodies [Balbis et al., 2004]. Thus, the integrity of the PM is severely affected at high doses of MBCD. In keeping with this view, the treatment of NIH 3T3 cells with 7.5 mM MBCD for 30 min not only increased EGF binding but also inhibited internalization and degradation of the EGFR, suggesting that non-raft domains were significantly affected [Pike and Casey, 2002].

In contrast to the above a recent study by Kazazic et al. [2006] showed that 1 mM MBCD had a minimal effect on EGF binding to and internalization of the EGFR. At this concentration of MBCD cholera toxin B internalization was inhibited but TfR internalization was unaffected suggesting that there was selective disruption of raft domains [Kazazic et al., 2006]. These results contrast with those using higher concentrations of MBCD [Pike and Casey, 2002; Ringerike et al., 2002; Roepstorff et al., 2002], and argue that these latter findings are attributable to effects on EGFR in non-raft domains.

Finally the biochemical methodology utilized for isolation of membrane rafts should be considered when comparing the results of different studies. We recently compared three methods to prepare low buoyant density membrane rafts from highly purified rat liver PM [Wang et al., 2009]. Two of these methods were detergent free ($NaCO_3$, pH 11 and OptiPrep) [Smart et al., 1995; Song et al., 1996], and the third used extraction with 1%Triton X-100 [Brown and Rose, 1992] to generate DRMs. PM DRMs displayed the highest enrichment of caveolin, and the absence of the TfR receptor in the rafts fraction [Wang et al., 2009]. The two detergent free methods showed a slight enrichment of caveolin but the amount of TfR present in these rafts fraction was equivalent to that found in the original PM, demonstrating substantial contamination with non-raft PM components [Wang et al., 2009].

In summary, the isolation of membrane rafts with methods that do not include Triton X-100 in the extraction plus the use of cholesterol sequestering drugs suggest that membrane rafts exert an inhibitory effect on EGFR activation and signaling. However, studies with Triton X-100 are in agreement that DRMs contain a small proportion of EGFR which is highly competent for signaling.

INTRACELLULAR MEMBRANE RAFTS

Most studies have focused on the role of membrane rafts at the plasma membrane. However, there is now increasing evidence for the presence of rafts in intracellular membranes [Helms and Zurzolo, 2004]. SV40 was shown to be internalized through caveolae into ENs in which caveolin content was maintained (caveosomes) [Pelkmans et al., 2001, 2004]. Caveosomes were identified as a subset of endocytic vesicles of neutral pH which were devoid of clathrin and other endosomal markers [Pelkmans et al., 2004]. However, caveolin is not required for the intracellular trafficking of virus since cells devoid of caveolin still internalized SV40 virus which was sorted to intracellular vesicles lacking caveolin [Damm et al., 2005]. Cholera toxin B can also exploit different mechanisms of internalization and intracellular trafficking that do not involve clathrin and may or may not involve caveolae [Pelkmans et al., 2004; Kirkham et al., 2005].

The non-caveolin, non-clathrin internalization pathway (s) are poorly understood. They all share a dependence on cholesterol but can be further classified by their dependence or independence on one or more proteins (viz. Cdc-42, Rho-A, and dynamin) [Doherty and McMahon, 2009; Hansen and Nichols, 2009]. Recently flotillin-1 has been proposed as a marker for caveolae and clathrin independent but raft dependent endocytosis [Glebov et al., 2006].

INTRACELLULAR TRAFFICKING AND SIGNALING OF THE EGFR: ROLE OF MEMBRANE RAFTS

EGF binding to EGFRs results in rapid internalization and concentration of the receptor in intracellular compartments. Though initially viewed as a mechanism for attenuating EGF signaling [Wells et al., 1990] it was soon apparent that EGF induced the recruitment and concentration of activated EGFR and signaling molecules in endosomes [Di Guglielmo et al., 1994; Sorkin et al., 2000]. Furthermore, Wang et al. [2002] showed that selective

activation of the endosomal EGFR resulted in the full range of downstream signaling and both cell proliferation and survival. However, the spatial control of EGFR signaling may not only depend on sorting to specific intracellular organelles but also on the segregation of signaling complexes to sub compartments (viz. rafts) within a given organelle.

In recent work Sigismund et al. [2008] showed that, at low EGF concentrations, the EGFR underwent clathrin mediated endocytosis and recycling back to the PM. At high concentrations EGFRs were internalized through a non-clathrin raft-dependent mechanism to late endosomes and lysosomes [Sigismund et al., 2008]; whereas the disruption of rafts (filipin treated cells) resulted in clathrin-dependent internalization and recycling [Sigismund et al., 2008]. These results suggested that endosomal membrane rafts are involved in sorting EGFRs to late endosomes–lysosomes for degradation. Fivaz et al. [2002] showed that the trafficking of GPI-anchored proteins to late endosomes correlated with its association with intracellular rafts, suggesting that membrane rafts are present in late endosomes. Sobo et al. [2007] showed that both the intra luminal and limiting membrane of late endosomes contain raft micro-

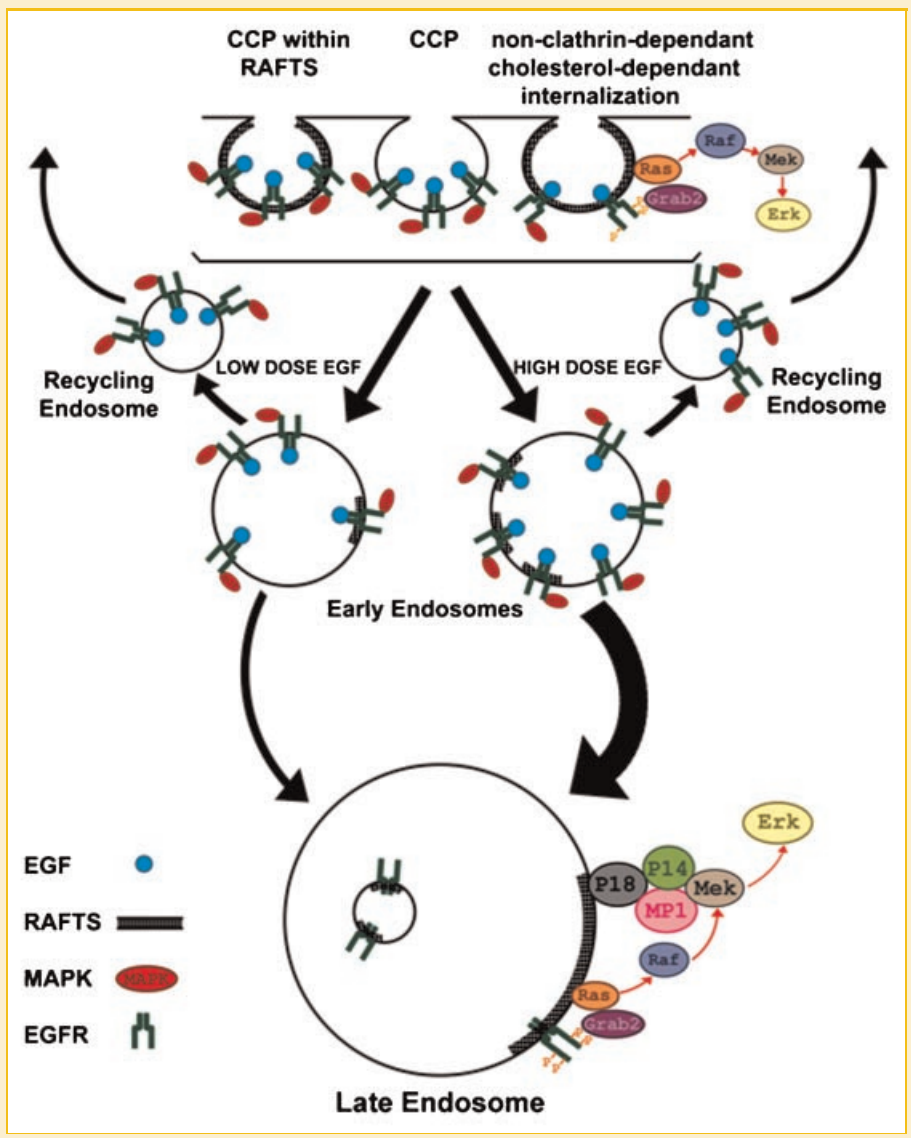

Fig. 1. Upon EGF binding at the PM the EGFR is activated resulting in the translocation and activation of MAPK components. The activated receptor is subsequently internalized into early endosomes. Previous work suggests that the EGFR is internalized mainly through a clathrin dependent raft-independent mechanism [Kazazic et al., 2006], and according to Puri et al. most of the EGFR is internalized from CCP that form within membrane rafts [Puri et al., 2005]. Sigismund et al. propose that at low doses of ligand the EGFR is internalized via a clathrin-dependent, cholesterol-independent pathway [Sigismund et al., 2005, 2008]. EGFR internalized through this mechanism is recycled back to the PM [Sigismund et al., 2008]. However, at high doses of EGF the internalization of the EGFR through a non-clathrin-dependent, cholesterol-dependent mechanism is increased [Sigismund et al., 2008; Wang et al., 2009] with increased, cholesterol-dependent trafficking of EGFR to late endosomes [Lai et al., 1989b; Sigismund et al., 2008]. In late endosomes and lysosomes the EGFR is degraded but recent work indicates that membrane rafts in late endosomes constitute an important signaling platform [Teis et al., 2002; Balbis et al., 2007; Taub et al., 2007; Nada et al., 2009] wherein hyper activated EGFR [Balbis et al., 2007], and the raft adaptor p18 are localized. This adaptor is necessary for anchoring the EGF-induced MAPK signaling complex to late endosomes [Teis et al., 2002; Teis and Huber, 2003; Nada et al., 2009].

domains which differ in composition and properties. Thus, late endosomes seem to contain membrane rafts that may be involved in the trafficking and subsequent degradation of the EGFR.

In agreement with these results, we found that high, but not low, doses of EGF reduced by 80% the content of EGFR in DRMs isolated from liver PM, suggesting rapid internalization of EGFR from membrane rafts [Wang et al., 2009]. Furthermore, we found that following EGF treatment DRMs isolated from both early and late endosomes, as well as PM, contained a distinctive population of EGFRs characterized by both a high level of Tyr-phosphorylation and augmented recruitment of Shc and Grb2 [Balbis et al., 2007; Wang et al., 2009]. Using magnetic beads to isolate intracellular vesicles enriched in Flotillin-1 or caveolin-1, we found that only the former contained EGFR that were highly Tyr-phosphorylated [Balbis et al., 2007] suggesting that rafts in endosomes devoid of caveolin function as signaling platforms [Balbis et al., 2007; Wang et al., 2009]. These intracellular rafts might correspond to the recently described endocytic intermediates that contain Flotillin-1 and accumulate GPI-anchored proteins and cholera toxin B [Glebov et al., 2006; Frick et al., 2007; Riento et al., 2009]. The augmented level of Tyr-phosphorylation of EGFRs in late endosomal DRMs when those in non-raft domains were largely dephosphorylated may result from restricted access of the former to endosomal Tyrosine phosphatases [Balbis et al., 2007].

If EGFR can signal from late endosomal rafts, what signaling pathway is linked to this pool of hyper active receptors? Evidence of EGF-induced late endosomal signaling comes from the work of Teis et al. [2002] who showed that the adaptor protein p14 is required to localize the MP1-MAPK signaling complex to late endosomes. Importantly, the localization of this protein complex in late endosomes is essential for proper EGF-induced MAPK signaling [Teis et al., 2002; Teis and Huber, 2003]. This is an interesting example of spatial regulation of the signaling cascade as knocking down p14 inhibited the late (endosomal) but not the early (plasma membrane) phase of MAPK activation [Teis et al., 2002; Teis and Huber, 2003]. We therefore suggest that the presence of hyper activated EGFR in late endosomal rafts [Balbis et al., 2007] is necessary for the recruitment and activation of the MAPK cascade in this compartment. In agreement with this hypothesis, Nada et al. [2009] recently showed that the novel membrane raft adaptor, p18, anchors the p14-Mp1-MEK1 complex to late ENs and is essential for EGF-induced MEK/Erk signaling. Consistent with the foregoing, Taub et al. [2007] recently showed that the spatial and temporal regulation MAPK signaling depended on the appropriate intracellular trafficking of the EGFR.

SUMMARY AND CONCLUSION

EM and biochemical techniques have established the presence of EGFRs in membrane rafts. High doses of EGF promote the sorting of EGFR to late endosomes through a raft/cholesterol dependant mechanism, implicating them in EGFR degradation. However, our work and that of others suggest that membrane rafts in late ENs participate in the spatial control of EGF-induced MAPK activation as depicted in Figure 1. Important questions remain. First are the intracellular rafts involved in trafficking of EGFR the same as those involved in signaling? Does specific disruption of intracellular rafts inhibit the late (endosomal) phase of EGF-induced MAPK activation? Does knock down of flotillin-1 interfere with endosomal trafficking of EGFR and activation of MAPK in late endosomes? No doubt a more complete picture of the role of membrane rafts in EGFR trafficking and signaling will emerge in the near future.

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