Emerging roles of recycling endosomes

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Cells internalize extracellular solutes, ligands and proteins and lipids in the plasma membrane (PM) by endocytosis. The removal of membrane from the PM is counteracted by endosomal recycling pathways that return the endocytosed proteins and lipids back to the PM. Recycling to the PM can occur from early endosomes. However, many cells have a distinct subpopulation of endosomes that have a mildly acidic pH of 6.5 and are involved in the endosomal recycling. These endosomes are dubbed recycling endosomes (REs). In recent years, studies have begun to reveal that function of REs is not limited to the endosomal recycling. In this review, I summarize the nature of membrane trafficking pathways that pass through REs and the cell biological roles of these pathways.

Keywords: endocytosis/exocytosis/phosphatidylserine/recycling endosomes/retrograde transport.

Abbreviations: CI-MPR, cation-independent mannose 6-phosphate receptor; CTxB, cholera toxin B subunit; EEs, early endosomes; HRP, horseradish peroxidase; LEs, late endosomes; PM, plasma membrane; PH, pleckstrin homology; PS, phosphatidylserine; REs, recycling endosomes; SM, sphingomyelin; Tfn, transferrin; TfnR, transferrin receptor; TGN, trans-Golgi network; TLR4, Toll-like receptor 4; TNF-α, tumour necrosis factor α; VSV-G, vesicular stomatitis virus glycoprotein G.

Role of REs in exocytic transport

The trans-Golgi network (TGN) has been the presumed sorting site for all newly synthesized membrane and secretory proteins (20, 21). A variety of experiments all consistently showed segregation of cargoes upon exit from the TGN. However, such data did not exclude the possibility that at least some sorting can take place at later compartments, for instance, in endosomes. From the late 1980s to 2000, there were a number of important suggestions in mammalian cells that exocytic cargo proteins can, at least to some extent, traverse endocytic compartments en route to the PM (22–25). For example, Futter et al. (23) showed that transferrin receptor (TfnR) was targeted first from the TGN to endosomes and subsequently delivered to the PM.

In the middle of 2000s, Ang et al. (26) provided direct evidence using four independent methods that REs play a role in the exocytic transport of vesicular stomatitis virus glycoprotein G (VSV-G) in the non-polarized stage of MDCK cells. By video microscopy,
immunoelectron microscopy and cell fractionation, newly synthesized VSV-G was found to enter Tfn-positive REs within a few minutes after exit from the TGN. Although transient, the entry into REs appeared essential because VSV-G delivery to the PM was impaired by enzymatic inactivation of REs with Tfn conjugated to horseradish peroxidase (HRP).

The list of exocytic proteins that traverse through REs before their delivery to the PM is expanding (Table 1). In HeLa cells, E-cadherin exited the Golgi in tubulovesicular carriers, which, instead of moving directly to the PM, most frequently fused with REs positive for Rab11 (a small GTPase) (27). In RAW 264.7 cells, tumour necrosis factor α (TNF-α) was trafficked from the Golgi to REs positive for VAMP3 (a v-SNARE) and Tfn, then to the PM at the site of phagocytic cup formation (28). Importantly, knockdown of VAMP3 impaired the PM delivery of TNF-α. In FRT cells in the presence of antibody against AP-1B, an epithelial-specific clathrin adaptor protein complex (29, 30) TfnR exited the TGN normally but became blocked at REs within 3–5 min (31). In polarized MDCK cells, the basolateral delivery of VSV-G was significantly suppressed when REs were enzymatically inactivated with Tfn-HRP (32). In COS-1 cells (33), palmitoylated H-Ras protein was transported from the TGN to REs and subsequently delivered to the PM (Fig. 2 (34)). H-Ras has two palmitoyl groups at C181 and C184. A monopalmitoylation mutant H-Ras-C184S was trafficked directly from the Golgi to the PM, while the other monopalmitoylation mutant H-Ras-C181S localized mostly in the Golgi. Therefore, both palmitoyl groups were found essential for the post-Golgi traffic of H-Ras through REs to the PM.

Roles of REs in retrograde transport

Retrograde transport is a process in which proteins and lipids are transported back from the PM/endosomes to the Golgi, and even to the ER (5, 6). Several Golgi proteins, such as TGN38/46, GP73 and furin, routinely escape the Golgi and are retrieved back through retrograde pathways (35–38). A membrane-anchored growth factor, HB-EGF, is targeted to the inner nuclear membrane from the PM after exposure of cells to shedding stimuli (39). Some protein toxins produced by bacteria and plants (such as cholera toxin, Shiga toxin and ricin) exploit this retrograde transport to reach the Golgi/ER, from where they are translocated to the cytosol to exert their cytotoxicity (40–42). The list of proteins that use retrograde transport is expanding rapidly, as is the number of their functions.

To reach the Golgi, retrograde cargoes internalized from the PM must pass through some combinations of EEs, LEs and/or REs. What has been clear is that the routes followed by retrograde cargoes vary depending on the cargo. For example, Tac-tagged furin is transported through EEs to LEs en route to the Golgi, whereas Tac-TGN38, which also cycles between the Golgi and the PM, is transported through EEs to REs en route to the Golgi (43). A more complex route

### Table 1. Examples of proteins that pass through REs.

<table>
<thead>
<tr>
<th>Cargo</th>
<th>Cellular function</th>
<th>Role of trafficking in cargo function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TfnR</td>
<td>Iron uptake</td>
<td>Receptor recycling from REs to the PM</td>
</tr>
<tr>
<td>LDLR</td>
<td>LDL/cholesterol uptake</td>
<td>Receptor recycling from REs to the PM</td>
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<tr>
<td>Glut4</td>
<td>Glucose uptake</td>
<td>Exocytic delivery to the PM</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Virus envelop</td>
<td>Exocytic delivery to the PM</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Cell adhesion</td>
<td>Exocytic delivery to the PM</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Cytokine</td>
<td>Exocytic delivery to the PM</td>
</tr>
<tr>
<td>H-Ras</td>
<td>Cell signalling</td>
<td>Exocytic delivery to the PM</td>
</tr>
<tr>
<td>TGN38, TGN46</td>
<td>Unknown</td>
<td>Steady-state localization at the Golgi</td>
</tr>
<tr>
<td>GP73</td>
<td>Unknown</td>
<td>Steady-state localization at the Golgi</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Growth factor</td>
<td>Translocation from the PM to the nuclear membrane</td>
</tr>
<tr>
<td>Shiga toxin, cholera toxin</td>
<td>Unknown</td>
<td>Translocation from the PM to the cytosol</td>
</tr>
<tr>
<td>CI-MPR</td>
<td>Lysosomal enzyme trafficking</td>
<td>Receptor recycling from endosomes to the TGN</td>
</tr>
<tr>
<td>TLR4</td>
<td>Innate immunity</td>
<td>Delivery to phagosomes</td>
</tr>
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may be followed by cation-independent mannose 6-phosphate receptor (CI-MPR), which, at steady state, cycles between the Golgi, PM and LEs. Kinetic evidence suggested that CI-MPR internalized from the PM is transported through EEs to REs en route to the Golgi (44). Retrieval of CI-MPR from LEs to the Golgi appeared to follow a TIP47/Rab9-dependent pathway from LEs (45).

McKenzie et al. (46) examined in detail if the passage through REs is required for a few retrograde cargoes in BSC-1 cells. When REs were enzymatically inactivated with Tfn-HRP, the retrograde transport to the Golgi of Shiga toxin and CI-MPR, but not that of furin, was severely impaired. Uchida et al. (47) showed that the retrograde traffic of cholera toxin B subunit (CTxB) was impaired at REs in COS-1 cells depleted of evectin-2, an RE protein with a pleckstrin homology (PH) domain (Fig. 3). The Golgi localizations of TGN38 and GP73 were also compromised by evectin-2 depletion, demonstrating that CTxB, TGN38 and GP73 traverse through REs en route to the Golgi.

One protein complex that is particularly important for retrograde traffic is the retromer (48, 49). The retromer allows cargo to be sorted away from EEs to the lysosomal degradation pathway. The mammalian retromer is a five-component complex, which is divided into two subcomplexes: one is composed of the mammalian homologues of yeast VPS26, VPS29 and VPS35, and the other is composed of two sorting nexin proteins. Although the retromer has been proposed to be engaged in direct retrograde transport from EEs to the

![Fig. 2 Exocytic transport of H-Ras through REs to the PM. The sequences of C-terminal 20-amino acid stretch of H- and N-Ras, palmitoylation-deficient mutants and Rap2 proteins are shown. After the exit from the Golgi, H-Ras is first transported to REs and then to the PM. A palmitoyl group on C181 (black) is required to exit the Golgi. Without the palmitoyl group on C184 (blue) upon the exit, mutant H-Ras (H-Ras-C184S) is directly transported to the PM.](https://academic.oup.com/jb/article-abstract/153/6/505/2182914)

![Fig. 3 Evectin-2 is essential for retrograde transport of CTxB to the Golgi. (A) Schematic illustration of organelle distribution in COS-1 cells. The Golgi exhibits a ring-shape appearance, and REs are spatially confined within the Golgi. EEs are excluded from inside the Golgi. (B) Fluorescent-labelled CTxB (in red) was taken up by COS-1 cells. After a 15-min chase, CTxB reached REs that are spatially encircled by the Golgi (GM130 in green). After a 75-min chase, CTxB reached the Golgi. A palmitoyl group on C181 (black) is required to exit the Golgi. Without the palmitoyl group on C184 (blue) upon the exit, mutant H-Ras (H-Ras-C184S) is directly transported to the PM. (C) The retrograde transport of CTxB from REs to the Golgi was significantly impaired in cells depleted of evectin-2. Even after a 90-min chase, CTxB was entrapped at REs. Scale bar, 10 μm.](https://academic.oup.com/jb/article-abstract/153/6/505/2182914)
Golgi, it remains possible that the retromer also functions in the retrograde pathway from EEs to REs.

Roles of REs in degradation transport

Transport pathways from REs to degradation organelles have recently been described. Toll-like receptor 4 (TLR4) is indispensable for recognition of Gram-negative bacteria. Husebye et al. (50) showed that endogenous TLR4 localizes mostly in Rab11-positive REs in human monocytes. By stimulation with *Escherichia coli*, TLR4 was transported from REs to *E. coli*-positive phagosomes. Knockdown of Rab11 resulted in emptying of TLR4 in REs, reduction of TLR4 around phagosomes and inhibition of *E. coli*-induced interferon regulatory factor-3 activation. Longatti et al. (51) showed that Rab11 promotes autophagy, a bulk degradation process characterized by the formation of double membrane vesicles called autophagosomes. They demonstrated a membrane transport pathway from REs to autophagosomes. Matsui et al. (52) showed that a small GTPase, Rab12, co-localizes with TfnR and Rab11. Knockdown of Rab12 in mouse embryonic fibroblast inhibited TfnR degradation without affecting the degradation pathway from EEs to lysosomes, suggesting a novel transport pathway from REs to lysosomes.

Determinants of RE localization

Although there has been little information about how proteins are targeted to REs, recent studies (palmitoylation of small GTPases and evectin-2) have shed light on this topic. Misaki et al. (34) showed that a 20-amino acid stretch at the C terminus of H-Ras or N-Ras that contains all the lipid modifications (farnesylation and palmitoylation) is sufficient for RE targeting (Fig. 2). H-Ras has palmitoyl groups at two cysteine residues (C181 and C184). By analysis of the subcellular localization of palmitoyl-deficient mutant H-Ras by introducing serine point mutations, both palmitoyl groups were found essential for the correct H-Ras targeting to REs. A monopalmitoylation mutant, H-Ras C181S, localized exclusively at the Golgi, whereas the other monopalmitoylation mutant, H-Ras C184S, localized at the Golgi and the PM. A null palmitoylation mutant, H-Ras C181/184S, accumulated mostly at the Golgi and a small amount localized at the ER. Interestingly, palmitoylation on C184 could be functionally replaced with L184, since a monopalmitoylation mutant, H-Ras C184L, localized at REs and the PM, in contrast to the localization of H-Ras C184S. Because L184 on N-Ras was found to be involved in N-Ras membrane binding (53, 54), palmitoyl groups and specific amino acid residues buried in membranes can function as the RE localization determinant. Uechi et al. (55) showed that three other small GTPases (Rap2A, Rap2B and Rap2C) also localize at REs. Rap2B is geranylgeranylated and Rap2A is farnesylated, and Rap2C is assumed to be farnesylated. All Rap2 proteins have two cysteines (C176 and C177) upstream of the CAAX cysteine (C180), and in Rap2B, C176 and C177 were demonstrated as the sites of palmitoylation (Fig. 2). The palmitoyl-null mutants of Rap2A, 2B and 2C did not localize to REs, showing that palmitoylation of Rap2 is essential for RE targeting, as is the case of H-Ras and N-Ras.

Uchida et al. (47) showed that phosphatidylserine (PS), a relatively minor constituent of biological membranes, is most concentrated in REs in COS-1 cells using a PS-specific probe. The enrichment of PS in REs was also demonstrated in other cell lines (56, 57). PS in REs was found essential for the RE localization of evectin-2, a regulator of retrograde traffic, through the binding of its PH domain to PS (47, 58).

Several studies indicated that REs have a unique lipid composition. Gagescu et al. (59) showed that REs purified from MDCK cells are highly enriched in the raft lipids [sphingomyelin (SM) and cholesterol] and PS. Mondal et al. (60) showed that sterols are enriched in REs using the fluorescent sterols. Yachi et al. (61) showed that SM is enriched in REs using a SM-binding protein, equinatoxin-II. Therefore, as is the case of evectin-2 that selectively binds PS through its PH domain, having interactions with these lipids may be one mechanism for RE targeting of proteins. Interestingly, affinity of palmitoyl groups for raft lipids has been demonstrated using *in vitro* reconstitution system (62).

Concluding remarks

Over the past decade, studies have shown that various cargoes, in addition to recycled ones, pass through REs during their travel to final destinations. Although much remains to be examined carefully as to whether the transit through REs is indeed required for individual cargoes, these studies suggest that REs serve as a sorting hub for multiple membrane trafficking pathways.

Many questions also remain unanswered regarding the molecular mechanisms underlying the transport through REs. For example, there is no clear model of how cargoes are sorted into distinct carriers, such as recycling and retrograde carriers. *In vitro* reconstitution systems, such as those developed for the early secretory pathway, and *in vivo* imaging systems to allow visualization of sorting process of multiple cargoes in REs would greatly benefit in our further understanding of the nature and regulators of the trafficking through REs. These studies are anticipated to make broad conceptual contributions, beyond the transports through REs, to the understanding of essentials of membrane traffic.

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Conflict of interest

None declared.
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