Endocytosis of lipid-anchored proteins: excluding GEECs from the crowd

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Endocytosis of glycosylphosphatidylinositol (GPI)-linked proteins via a specific pathway into GPI-enriched early endosomal compartments (GEECs) has been proposed. How sorting into this pathway may take place is unclear. In this issue, Bhagatji et al. (2009. *J. Cell Biol.* doi:10.1083/jcb.200903102) provide an original mechanism for the sorting of lipid-anchored proteins that involves exclusion of bulky extracellular domains from clathrin-coated pits.

Proteins attached to the outer leaflet of the plasma membrane by a GPI lipid anchor can be taken up into the cell via clathrin-independent endocytosis and may be concentrated in specialized endosomal compartments termed GEECs (Sabharanjak et al., 2002; Mayor and Riezman, 2004). However, many researchers in this area have been puzzled by the fact that despite intensive investigation, concentration of GPI-linked proteins in nascent endocytic intermediates has never been visualized. How membrane proteins that lack cytoplasmic domains are sorted during endocytosis has been unclear. Innovative experiments published in this issue address these topics (see Bhagatji et al. on p. 615 of this issue). The new data imply that sorting of lipid-anchored proteins is more strongly influenced by steric exclusion of bulky protein moieties from the presumptively crowded environment of the clathrin-coated pit than by specific properties of the lipid anchor.

Understanding the mechanism by which GPI-linked proteins are sorted during endocytosis is important for several reasons. First, GPI-linked proteins have central roles in many different cell biological processes, ranging from nutrient uptake to complement fixation, and internalization may play a key part in these processes. Second, GPI-linked proteins are thought to be incorporated into small microdomains or lipid rafts in the plasma membrane (Simons and Ikonen, 1997; Sharma et al., 2004). The existence, size, and functional importance of these structures are under debate, and this debate will be informed by data on the dynamics and sorting of GPI-linked proteins (Munro, 2003; Mayor and Riezman, 2004). Finally, GPI-linked proteins are archetypal cargoes for clathrin-independent endocytosis, so experiments that alter the rate of uptake of such proteins may be interpreted as reflecting changes in the activity of specific sets of endocytic machinery (Mayor and Riezman, 2004; Naslavsky et al., 2004; Glebov et al., 2006; Mayor and Pagano, 2007; Lundmark et al., 2008).

The term GEEC was coined in a key paper from Sabharanjak et al. (2002). This paper showed that the folate receptor and other GPI-linked proteins are internalized into a population of endosomes that are not labeled with transferrin, which is the model cargo for clathrin-dependent endocytosis. Furthermore, the internalization of GPI-linked proteins does not require clathrin-coated pit or dynamin GTPase function. A chimeric protein constructed by placing the extracellular protein domain of the folate receptor onto a heterologous transmembrane domain was excluded from the endosomes containing GPI-anchored proteins. These data lead to the related hypotheses that GPI-linked proteins are enriched in GEEC endosomes over other membrane proteins and that the lipid anchor of GPI-linked proteins plays an important role in sorting into GEECs.

Bhagatji et al. (2009) designed experiments with the explicit goal of determining the role of lipid anchoring in sorting into GEECs. They have developed artificial phosphatidylethanolamine-polyethyleneglycol (PE-PEG) anchors conjugated to different extracellular moieties and shown that these can be incorporated into the outer leaflet of the plasma membrane (Wang et al., 2005). As the PE-PEG anchors can be synthesized with different acyl chains, the influence of both of the acyl chains and the nature of the extracellular conjugates on endocytic sorting could be ascertained (Fig. 1). Remarkably, three different proteins attached to PE-PEG anchors were endocytosed along with GPI-linked folate receptors to the GEEC compartment, regardless of anchor acyl chain length and saturation. In this case, this implies that the nature of the lipid acyl chains is not a significant factor in targeting lipid-anchored proteins to GEECs and that specific properties of the GPI anchor itself are not necessary for entry into the GEEC pathway. How then can one account for the apparent sorting of both GPI-linked proteins and the exogenous PE-PEG–anchored proteins away from clathrin-coated pits and into GEECs? An intriguing answer to this puzzle is suggested by further experiments in which the same PE-PEG anchors were coupled to a small fluorophore rather than to

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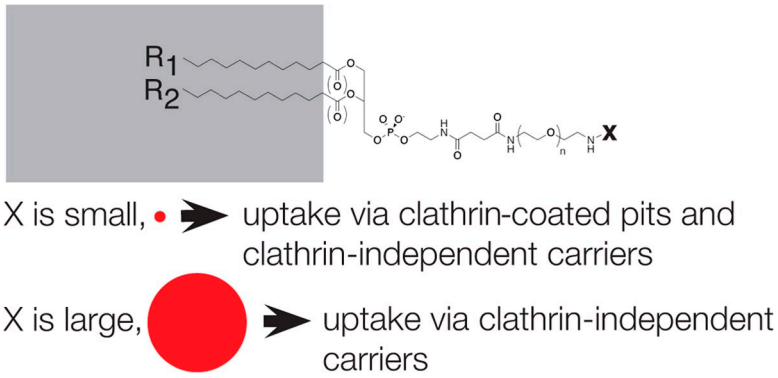

Figure 1. **Artificial lipid anchors.** The figure depicts an example of a PE-PEG membrane anchor used by Bhagatji et al. (2009). The gray box denotes the portion of the molecule embedded in the plasma membrane. Variation in the acyl chains R_1 and R_2 did not affect endocytic sorting. When a small head group X (such as tetramethylrhodamine) was used, the PE-PEG could enter clathrin-coated pits, but when X was large (such as dihydrofolate reductase bound to PE-PEG–methotrexate), colocalization with GPI-linked proteins in clathrin-independent carriers was observed. The red circles give an approximate indication of the relative sizes of tetramethylrhodamine and dihydrofolate reductase based on molecular weight.

relatively large proteins. This reduction in conjugate size was sufficient to allow incorporation into clathrin-coated pits, as judged by colocalization with transferrin after short times of uptake (Fig. 1). All of this implies the model shown in Fig. 2. In the model, the size of the extracellular protein moiety is a critical parameter for endocytic sorting of lipid-anchored proteins. Furthermore, the most significant factor in determining sorting to different endocytic pathways is simply the fact that the clathrin-coated pit, where multiple cargo proteins are very efficiently concentrated, is a crowded environment in which steric factors lead to the exclusion of bulky proteins that lack specific sorting signals. Lipid-anchored proteins do not then need to be effectively concentrated within the plane of the plasma membrane for apparently selective entry into clathrin-independent endocytic pathways (exclusion from coated pits is enough).

How can the model shown in Fig. 2 be reconciled with the data of Sabharanjak et al. (2002), in which substitution of the lipid anchor of the folate receptor with a transmembrane domain lead to exclusion from GEECs? Both the study of Bhagatji et al. (2009) and that of Sabharanjak et al. (2002) used the same cell line and markers. One can suggest two speculative explanations. First, the answer may lie in the exact nature of the transmembrane construct used by Sabharanjak et al. (2002). The folate receptor was fused to a chimeric protein containing a transmembrane domain from Fc-γ receptor and the cytosolic domain of low density lipoprotein (LDL) receptor with key residues required for recruitment to clathrin-coated pits mutated. As the LDL receptor has been reported to dimerize via its cytosolic domain (van Driel et al., 1987), this chimera could possibly be recruited to coated pits by interacting with endogenous LDL receptors, or there could be some other unforeseen property of the chimera that means it does not function as a sorting-neutral transmembrane domain. Second, the nature of the endocytic carriers involved in the GEEC pathway could lead to exclusion of transmembrane domains from these carriers without an active concentration of GPI anchors (Fig. 2). The relevant carriers have not been fully characterized, but two different classes of proteins associated with clathrin-independent endocytosis, caveolins and flotillins, are embedded in the cytosolic face of the plasma membrane and oligomerize to generate membrane microdomains (Rothberg et al., 1992; Stuermer et al., 2001; Bauer and Pelkmans, 2006; Frick et al., 2007). It is possible that such oligomerization could exclude transmembrane domains but not lipid anchors in the outer leaflet of the membrane.

The experiments of Bhagatji et al. (2009) have important implications. They provide an explanation for the observed endocytic sorting of GPI-linked proteins that does not need to invoke specific properties of the lipid anchors of these proteins or incorporation into raft-like lipid microdomains. They suggest that the term GEEC may be something of a misnomer, as there is as yet no direct evidence that the density of GPI-linked proteins per unit area of membrane is any higher in the GEEC

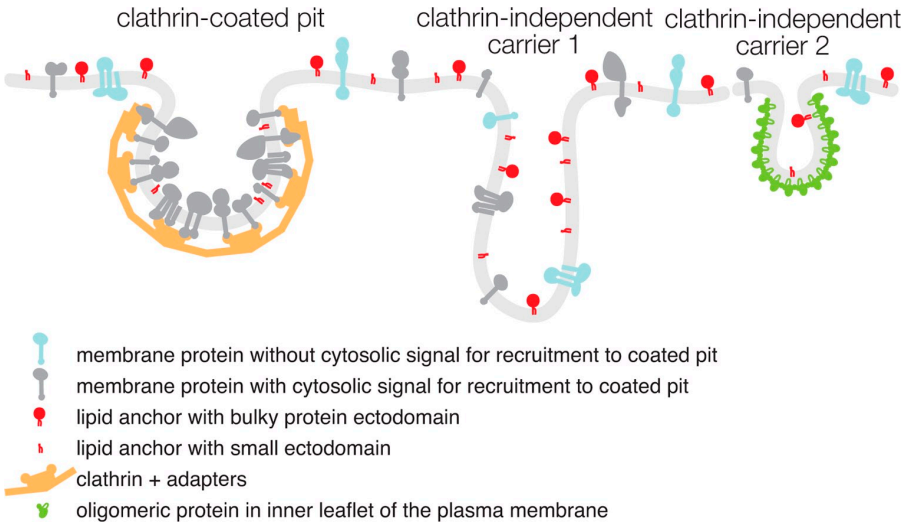

Figure 2. **Ectodomain size determines the endocytic pathway for lipid-anchored proteins.** The results of Bhagatji et al. (2009) suggest a model in which lipid-anchored proteins are excluded from clathrin-coated pits when they have a bulky ectodomain. In endocytosis via clathrin-independent carrier 1, lipid-anchored proteins are present at the same density in the rest of the plasma membrane. In the speculative clathrin-independent carrier 2, which is generated by oligomerization of a protein embedded in the cytosolic face of the plasma membrane (analogously to caveolins or flotillins), transmembrane domains are excluded. This could lead to selectivity for lipid-anchored proteins in the outer leaflet of the membrane without this latter class of protein being actively concentrated.

compartments than in the plasma membrane. And they imply that if GPI-linked proteins are sorted chiefly by exclusion from coated pits, they may enter the cell by multiple clathrin-independent endocytic pathways. Direct visualization of the endocytosis of GPI-linked proteins in carriers or vesicles defined by specific markers would clearly help to resolve these issues.

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