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Intracellular transport of GPI-anchored proteins

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In eukaryotic cells, a subset of proteins are attached to the external leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. There is substantial evidence suggesting that these GPI-anchored proteins are clustered in sphingolipid–sterol microdomains or rafts. Since the precursors of these microdomain components are synthesized mainly in the endoplasmic reticulum, it is possible that microdomain assembly occurs during transport along the exocytic route. A sorting mechanism for GPI-anchored proteins using sphingolipid microdomains as selective platforms for vesicle budding has been proposed to operate at different steps in the secretory pathway. Here, we discuss this sorting model in the context of the data obtained from different biological and artificial systems, in addition to other particularities of the intracellular transport of the GPI-anchored proteins.
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Introduction

The regulation of intracellular protein and lipid transport is one of the central issues in cell biology. However, the mechanisms regulating the sorting process that operate to target newly synthesized proteins and lipids from their site of synthesis, the endoplasmic reticulum (ER), to their correct final destinations are only partially understood (Rothman and Wieland, 1996; Schekman and Orci, 1996). It is clear that many proteins, transported along the secretory pathway, contain specific sorting signals. These signals can be recognized by sorting receptors and can be used for the forward transport of the protein or for its retrieval to the compartment from which it exits [i.e. mannose 6-phosphate receptor (Kornfeld, 1992) and KDEL receptor (Pelham, 1995)]. In addition, interaction of proteins with lipids can provide a different type of sorting mechanism. Membranes along the secretory pathway have different lipid compositions. This heterogeneity can be explained by differential localization of lipid biosynthetic enzymes and by a preferential transport of specific lipids from their site of synthesis to other destinations. Generation of differences in lipid composition by vesicular trafficking also involves the lateral heterogeneity of lipids. Since lipids can be transported with membrane proteins via transport vesicles, it has been proposed that specific lipid domains can play a role in the sorting of membrane proteins (Bretscher and Munro, 1993; Simons and Ikonen, 1997).

GPI-anchored proteins can be clustered in sphingolipid–cholesterol microdomains or rafts

The intracellular transport of glycosylphosphatidylinositol (GPI)-anchored proteins depends on specific lipids such as sphingolipids and cholesterol. At the present time, this seems to be the most defined system to study lipid-mediated protein sorting. The basic components of this system are sphingolipids, cholesterol and GPI-anchored proteins (in addition to some transmembrane proteins and double acylated tyrosine kinases of the Src family kinases; Simons and Ikonen, 1997).

GPI-anchored proteins are attached to the external surface of the plasma membrane of eukaryotic cells by their GPI moiety and they play a wide variety of physiological roles, including transmembrane signaling, cell surface protection, cell adhesion and cell wall synthesis. The typical GPI anchor structure, with a core structure EthN-P-Man_3-GlcN-PI, and its biosynthetic pathway are mostly conserved between organisms. Once synthesized, the GPI anchor is added *en bloc* by transamidation to the C-terminal carboxyl group of a protein. This process occurs in the lumen of the ER, where the protein to be anchored had been cleaved previously near the C-terminus. GPI anchors can be modified on different mannose residues and, in general, the lipid moiety is often replaced in a remodeling reaction by another lipid containing a highly saturated acyl chain (for general reviews of GPI-anchored protein synthesis, see Englund, 1993; Ferguson, 1999). Newly synthesized GPI-anchored proteins leave the ER in COPII-coated vesicles and travel via the Golgi to their final destination, the plasma membrane (Doering and Schekman, 1996; Sütterlin *et al*., 1997).

Sphingolipids are ceramides with a polar head group. Ceramide is made in the ER when a saturated long chain fatty acid is added to a long chain base. This molecule can be modified further by hydroxylation and receives the polar head group after transport to the Golgi. Sphingolipids are transported from the Golgi to the plasma membrane, being concentrated in the exoplasmic leaflet like the GPI-anchored proteins (for a review, see Dickson, 1998).

Sterols, like ceramide, are synthesized mainly in the ER, and are transported to and accumulated in the plasma membrane. However, they can be found in intracellular membranes such as the Golgi, ER, nucleus, lysosomes and mitochondria, and in lipid particles. Basically, sterol molecules consist of a tetracyclic hydrocarbon ring system with a planar conformation. Sterols apparently reside adjacent to the hydrocarbon chains of sphingolipids and

glycerolipids, closer to the aqueous interface than to the bilayer midplane (Brown, 1998; Daum *et al*., 1998).

Sphingolipids have been proposed to be in a liquid-ordered (Lo) phase, since they have a high melting temperature. In contrast, glycerolipids, with a lower melting temperature, seem to be in a liquid-disordered (Ld) phase. In cellular membranes, this difference could produce a lateral association of sphingolipids, creating a Lo phase, microdomain or raft, surrounded by glycerolipids disposed in a more fluid Ld phase. This idea is consistent with the fact that sphingolipids are found in cold detergent-insoluble extracts (Brown and London, 1998). The interpretation of this result is that sphingolipids are associated laterally *in vivo*. Although the possibility that this association may be an artifact of the extraction procedure cannot be excluded, recent evidence suggests that this insolubility reflects physical association *in vivo* (Ahmed *et al*., 1997; Sheets *et al*., 1997; Schroeder *et al*., 1998). In addition, a non-detergent-based technique, fluorescence quenching, shows that sphingolipid Lo phase formation occurs in artificial membranes and that this formation correlates well with detergent insolubility (Ahmed *et al*., 1997). However, there is no direct evidence for the existence of sphingolipid-containing Lo phase domains in living cells.

It has been proposed that the clustering of sphingolipids is due to the creation of a hydrogen bond network (Simons and van Meer, 1988). Although there is evidence for a role for the hydrogen bonds, it seems that the interaction among the saturated acyl chains is the main factor promoting clustering of sphingolipids (Schroeder *et al*., 1994). The fact that glycerolipids contain unsaturated acyl chains reinforces this idea.

In mammalian cells, cholesterol seems to form part of the sphingolipid microdomains because it is found in the cold detergent-insoluble extracts (Brown and Rose, 1992). In addition, there is evidence suggesting that cholesterol associates more readily with sphingolipids than with glycerolipids (Sankaram and Thompson, 1990), although it can also associate with glycerolipids (Brown, 1998). In principle, cholesterol does not appear to be essential for sphingolipid microdomain formation, but it can increase the packaging of the sphingolipids (Smaby *et al*., 1996). It has also been proposed that small amounts of cholesterol might localize along the border between sphingolipid rafts and glycerolipids. This arrangement of cholesterol could create an energy-favorable transition area between these two different phases (Cruzeiro-Hansson *et al*., 1989). Thus, cholesterol could regulate the physical properties of sphingolipid microdomains by interacting with the saturated acyl chains of sphingolipids, inside and along the border of the raft (Simons and Ikonen, 1997; Brown, 1998).

Since GPI anchors contain a saturated acyl chain, GPI-anchored proteins would tend to be associated with sphingolipids rather than glycerolipids. The following evidence supports this idea. (i) GPI-anchored proteins are found together with sphingolipids and cholesterol in cold detergent-insoluble extracts from cells and from liposomes (Brown and Rose, 1992; Schroeder *et al*., 1994). (ii) Saturated acyl chains in the sphingolipids are required for the presence of GPI-anchored proteins in cold detergent-resistant extracts (Schroeder *et al*., 1994). (iii) The insolubility of GPI-anchored proteins depends on the abundance of sphingolipids and cholesterol in artificial lipid mixtures (Schroeder *et al*., 1998). Consistent with this, depletion of sphingolipids or cholesterol *in vivo* leads to the absence of GPI-anchored proteins in the detergent-insoluble extracts (Cerneus *et al*., 1993; Hanada *et al*., 1995). (iv) It has been shown that GPI-anchored proteins are organized in microdomains at the cell surface and that cholesterol is necessary for this organization (Stauffer and Meyer, 1997; Friedrichson and Kurzchalia, 1998; Harder *et al*., 1998; Varma and Mayor, 1998). This evidence also suggests that the GPI anchor might be a signal to target proteins to sphingolipid domains.

Although all of these data support the presence of the sphingolipid rafts in living cells, the issue is still debated. Assuming their existence, other questions such as their size or lifetime remain open. Theoretical studies and experimental data suggest that rafts are small and dynamic but can form larger structures such as caveolae for example (Rietveld and Simons, 1998).

Sorting of GPI-anchored proteins based on their association with the sphingolipid microdomains

All precursors of sphingolipid rafts are synthesized in the ER and have to be transported to the plasma membrane where they accumulate. Therefore, it is possible that assembly of the rafts and incorporation of the GPI-anchored proteins into them occur during the transport along the secretory pathway. Raft formation could operate as a sorting mechanism for GPI-anchored proteins by using the sphingolipid microdomains as selective platforms for vesicle budding (Simons and Ikonen, 1997). There are several studies that have tried to address this issue in different organisms.

In mammalian cells, it has been observed that when the synthesis of ceramide (the precursor of the sphingolipids) is decreased by incubation with fumonisin (Wang *et al*., 1991), GPI-anchored proteins are specifically mis-sorted in MDCK cells and in primary hippocampal neurons (Mays *et al*., 1995; Ledesma *et al*., 1998). In addition, cholesterol is required for the selective transport of a GPI-anchored protein to the cell surface (Hannan and Edidin, 1996). These experiments are in agreement with the original model which suggests that, in mammals, sphingolipid microdomains recruit GPI-anchored proteins at the *trans*-Golgi network (TGN) and somehow determine which type of transport vesicle they enter; going to the basolateral or apical plasma membrane (Simons and van Meer, 1988).

In yeast, ceramide is required for specific transport of GPI-anchored proteins from the ER to the Golgi (Horvath *et al*., 1994; Skrzypek *et al*., 1997; Sütterlin *et al*., 1997). When yeast cells are incubated with myriocin, an inhibitor of ceramide synthesis, or when *lcb1* (serine palmitoyltransferase, the first enzyme in ceramide biosynthesis) mutant cells are incubated at non-permissive temperature, the ER to Golgi transport of the GPI-anchored protein Gas1p is defective. This ceramide requirement is specific for GPI-anchored proteins because the transport of non-GPI-anchored proteins is normal under these conditions. From these results, it has been proposed that, in yeast, GPI-anchored proteins may be sorted from non-GPI-

anchored proteins in the ER by selective recruitment in ceramide-rich microdomains.

This leads to an apparent discrepancy concerning the point in the secretory pathway where GPI-anchored proteins are incorporated into the ceramide/sphingolipid domains. In the mammalian model, the initial sorting occurs in the Golgi and, for the yeast model, sorting occurs in the ER. There are two possibilities to explain this difference. First, the sorting of GPI-anchored proteins may begin in the ER in both organisms. Secondly, GPI-anchored protein sorting could occur in different organelles depending on the organism due to different physical properties of ceramides, sphingolipids and sterols.

According to the first explanation, the proposed difference in the site of sorting between yeast and mammalian cells is not justified. In this case, the formation of the rafts could take place in the ER in both organisms. The sole evidence for the localization of the site of raft formation and GPI-anchored protein sorting in mammalian cells is that only mature GPI-anchored proteins can be found in the cold detergent-insoluble extracts (Brown and Rose, 1992). However, this experiment does not solve the problem of the microdomain location. Therefore, the possibility that ceramide microdomains are formed in the ER has not been excluded. It could be that microdomains in mammalian cells are formed in the ER, but are simply not cold detergent resistant. Thus, it is possible that cold detergent insolubility only reflects a further ordered state of a pre-formed microdomain. This state may only be reached in the late transport steps, where the concentration and packaging of all the components of the rafts are expected to be higher than in the membranes of the early secretory pathway. In fact, it has been shown that Triton X-100 insolubility underestimates the amount of Lo phases in artificial membrane systems (Schroeder *et al*., 1998). Therefore, it is plausible that Triton X-100 treatment cannot detect the ER rafts. The possibility cannot be excluded that by using detergents with other properties, the ER microdomains could become cold detergent resistant.

As mentioned above, the mis-sorting of GPI-anchored proteins observed after ceramide depletion agrees with the model of sorting in the Golgi. However, these studies did not investigate the kinetics of transport of GPI-anchored proteins from the ER to the Golgi. Thus it is possible that this step was affected. In addition, the use of inhibitors such as fumonisin and myriocin has limitations. Ceramide synthesis is only decreased, but not blocked, by these compounds. In yeast, when ceramide synthesis could be blocked efficiently using a temperature-sensitive mutant, the effect on ER to Golgi transport of GPI-anchored proteins was much stronger than with myriocin (Sütterlin *et al.*, 1997).

Sorting of GPI-anchored proteins in the Golgi mediated by sphingolipid microdomains does not exclude a sorting by ceramide microdomains in the ER. A ceramide microdomain formed in the ER could be maintained in the Golgi and used for a later sorting step. The higher concentration of cholesterol and the addition of head groups to form sphingolipids in the Golgi would provide more opportunities for hydrogen bonding and could increase the size, packaging and stability of the pre-formed ER rafts.

Finally, recent evidence shows a segregation of GPI biosynthetic reactions in a subcompartment of the ER in BW5147 mouse thymoma cell line (Vidugiriene *et al*., 1999). This is consistent with the idea that GPI anchors may already be segregated in specific domains of the ER in mammalian cells. In addition, this segregation is also present in *Leishmania mexicana*, and the specific ER subcompartment can be stained specifically with BODIPY-C5-ceramide (Ilgoutz *et al*., 1999). This supports the notion that ceramides and GPI anchors may be partitioned by a common mechanism in the ER.

If GPI-anchored protein sorting and microdomain formation truly takes place in different locations in animals and yeast, then this could result from differences in the structural particularities of GPI anchors and sphingolipids in the different organisms. One clear difference between mammals and yeast is the length of the acyl chains of sphingolipids (Dickson, 1998) and GPI anchors (McConville and Ferguson, 1993). In yeast, ceramide contains a very long saturated acyl chain (C26). This fact might favor the clustering of ceramides and the formation of thicker microdomains in the ER. There is evidence that the thickness of the membrane can be a sorting mechanism for membrane proteins (Munro, 1998). This could be the case for GPI-anchored proteins in yeast. The lipid moiety of the GPI anchor is exchanged from diacylglycerol to ceramide (C26) or to a more hydrophobic diacylglycerol with a very long chain fatty acid (C26). These remodeling events can occur in the ER (Sipos *et al*., 1997). Thus, the long acyl chains could act to sort the GPI-anchored proteins into the thicker ER ceramide microdomains and, consequently, drive the incorporation of these proteins into distinct ER-derived vesicles (Reggiori *et al*., 1997). Consistent with this hypothesis, *elo2* and *elo3* (fatty acid chain elongation machinery in yeast) mutants are defective specifically in transport of GPI-anchored proteins from the ER to the Golgi (David *et al*., 1998). In addition, mutants that can survive without sphingolipids do so by synthesizing novel long chain fatty acids (Nagiec *et al*., 1993).

In mammals, the acyl chains of ceramides (C18–C24) and GPI anchors are shorter than in yeast. Thus, the thickness-based sorting mechanism would not be predicted to take place in the ER. However, it could still occur in the Golgi (Munro, 1998), where the domains of the membrane containing sphingolipids can be thicker than in the ER, because cholesterol is more abundant there and sphingolipid is able to form thicker bilayers in the presence of cholesterol (Nezil and Bloom, 1992; Maulik and Shipley, 1996). Curiously, there is a correlation between the length of the ceramide acyl chains and the abundance of sterols. In nematodes and insects, which are auxotrophs for sterol and consequently have less sterol, the acyl chain of ceramides is longer than in mammalian cells, where the cholesterol is very abundant (Munro, 1998).

Another difference between yeast and mammals is that the hydroxylation of the fatty acid of ceramide is common in yeast but rare in mammals (Dickson, 1998). These polar groups could be utilized to form hydrogen bonds between adjacent ceramides or ceramides and GPI anchors. This could increase the efficiency of ER clustering of ceramides in yeast but not in mammals. Mammalian ceramides would then cluster in the Golgi where they

are converted to sphingolipids, whose head groups are involved in hydrogen bonds.

Additional requirements for GPI-anchored protein transport

In addition to the lipid-mediated sorting, transport of GPI-anchored proteins from the ER to the plasma membrane presents other specific characteristics. GPI anchoring has been proposed to be a requirement for exit from the ER. The experiments supporting this idea showed that when GPI anchoring was not completed in yeast microsomes, the unanchored proteins were not included into newly formed, ER-derived vesicles (Doering and Schekman, 1996). In trypanosomes, the GPI anchor also seems to be required for exit from the ER of a GPI-anchored protein (McDowell *et al*., 1998). However, the experiments in both systems did not differentiate between the GPI anchor acting as a positive signal and the remaining uncleaved C-terminus acting as a retention signal. In fact, mutation of the polar amino acids in the uncleaved portion of the C-terminus of a GPI-anchored protein precursor, that could not be anchored due to mutation of its anchor attachment site, allowed transport of the unanchored and uncleaved protein to the Golgi apparatus *in vivo* (Horvath *et al.*, 1994). These experiments are consistent with the uncleaved hydrophobic C-terminus acting as a retention signal. More incisive experiments are necessary to determine whether the GPI anchor can act as an ER exit or other targeting signal.

Several lines of evidence suggest that cargo proteins are incorporated selectively in the ER into COPII-coated vesicles to be transported to the Golgi (Mizuno and Singer, 1993; Balch *et al*., 1994; Kirchhausen *et al*., 1997; Herrmann *et al*., 1999). This sorting process is driven by the interactions between the secretory proteins and the COPII components (Aridor *et al*., 1998; Kuehn *et al*., 1998). GPI-anchored proteins are lumenal. Therefore, they are not able to interact directly with the COPII coat on the cytosolic face of the budding membrane. In this case, the COPII-dependent sorting model implies the existence of a transmembrane adaptor(s) to connect the lumenal GPI-anchored proteins with the cytosolic coat components. Emp24p and Erv25p, members of the p24 family in yeast, are good candidates to function as adaptors/receptors for the GPI-anchored protein, Gas1p. Both proteins form part of the same complex, they are major components of the COPII-coated vesicles and they are required for the efficient transport of Gas1p from the ER to the Golgi (Schimmoller *et al*., 1995; Belden and Barlowe, 1996). Two other members of the p24 family have also been shown to affect Gas1p transport from ER to Golgi and are proposed to associate with Emp24p and Erv25p (Marzioch *et al*., 1999). Recent direct evidence shows that Emp24p is required for the selective incorporation of a GPI-anchored protein, Gas1p, into ER-derived vesicles. It has been postulated that these proteins constitute part of a cargo receptor involved in ER exit, although additional roles for Emp24p, such as in microdomain formation or stabilization, cannot be ruled out (M.Muñiz, C.Nuoffer, H.P.Hauri and H.Riezman, submitted). These putative receptors may not be exclusive for GPI-anchored proteins, because the ER to Golgi transport of the soluble protein, invertase, is reduced *in vivo* in *emp24* and *erv25* mutants.

In yeast, the transport from the ER to the Golgi of GPI-anchored proteins is inhibited strongly in mutants of α-COP (*ret1-1*), a component of the COPI coat (Sütterlin *et al*., 1997). COPI has been shown to be involved in retrograde transport from the Golgi to the ER (Letourneur *et al*., 1994). Thus, it is conceivable that coatomer could function in the retrieval of some specific factor(s) from the Golgi to the ER required for the forward transport of GPI-anchored proteins (Sütterlin *et al*., 1997).

In many polarized epithelial cell lines, GPI-anchored proteins are localized mainly in the apical membrane. It was proposed previously that this apical delivery is due to the GPI anchor. However, more recent evidence suggests that the GPI anchor is not the apical sorting signal and that, as for other glycosylated proteins, *N*-glycans are used to direct GPI-anchored proteins to the apical membrane (Benting *et al*., 1999).

Perspectives

The model of the sorting of GPI-anchored proteins mediated by lipid microdomains is very attractive, but many open questions still remain to be addressed. One crucial question is whether microdomains can be detected in internal membranes such as the ER or the Golgi. The techniques used to study the association of GPI-anchored proteins in the plasma membrane could be powerful tools for that purpose. Microscale lipid analysis could also be very helpful to address this question. More functional studies are also necessary in order to establish the specific role of ceramides/sphingolipids, sterols and the GPI anchor in the intracellular transport of GPI-anchored proteins in different model organisms. Finally, it is necessary to obtain direct evidence that would be consistent with GPI-anchored protein sorting mediated by ceramide/sphingolipid microdomains. This could be done by analyzing the lipid and protein composition of transport vesicles derived from the ER or Golgi by using *in vitro* budding assays.

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