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Glycosylation of glycolipids in the Golgi complex

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Abstract

Gangliosides are a family of glycolipids characterized by containing a variable number of sialic acid residues. Nearly, all animal cells contain at least some class of ganglioside in their membranes, but membranes from the CNS are characterized by their high content of these lipids. The synthesis of the oligosaccharide moiety of glycolipids is carried out in the Golgi complex. In this study, I will discuss the cellular and molecular basis of the organization of the glycosylating machinery in the Golgi complex, with particular attention to the mutual relationships, sub-Golgi localization, and intracellular trafficking of glycolipid glycosyltransferases, and to their relationships with the corresponding glycolipid acceptors and sugar nucleotide donors. I will also discuss how the organization of the glycosylating machinery in the Golgi may adapt to events controlling glycolipid expression.

Keywords: endoplasmic reticulum exiting, ganglioside glycosylation, glycolipid synthesis, glycosyltransferase trafficking, golgi complex glycosylation, Sar1.

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Most glycolipid glycosyltransferases (GGT) acting in the synthesis of lipid-bound oligosaccharides are integral proteins of the Golgi complex membranes. GGT transfer sugar units in a stepwise manner from sugar nucleotide donors imported from the cytoplasm (Fig. 1a). Similarly to glycoprotein glycosyltransferases, GGT are Type II membrane proteins with an N-terminal domain (Ntd) consisting of a relatively short cytoplasmic tail facing the cytoplasm, a transmembrane uncleaved signal-anchor region, a stem region and a lumenally oriented C-terminal domain that bears the catalytic site and the sugar nucleotide-binding site (Paulson and Colley 1989; Colley 1997).

Glycosyltransferases that transfer sugars to ceramide show differences in localization and also in membrane insertion topology: the endoplasmic reticulum (ER) concentrated ceramide galactosyltransferase has a Type I topology, with a lumenal Ntd bearing an ER retrieval signal and the catalytic site (Schulte and Stoffel 1993; Sprong *et al.* 1998). Ceramide glucosyltransferase (GlcT), on the other hand, has a Type III topology, with N-terminal uncleaved signal anchor sequence and a long cytoplasmic tail bearing the catalytic site (Coste *et al.* 1986; Futerman and Pagano 1991; Jeckel *et al.* 1992). There is some debate on the subcellular localization of ceramide GlcT; in liver cells, it was reported to be widely distributed between Golgi and ER membranes (Futerman and Pagano 1991) but located in proximal and distal Golgi fractions by others (Jeckel *et al.* 1992). Immunocytochemical visualization of a tagged form of *Drosophila melanogaster* GlcT transfected to B19 melanoma cells lacking GlcT revealed its presence both in ER and Golgi membranes (Kohyama-Koganeya *et al.* 2004).

Functional and topological relationships of transfer steps

Initial studies on the ordering of GGT along the Golgi subcompartments were somehow influenced by the concepts emanating from studies on glycoprotein oligosaccharides synthesis and processing (Kornfeld and Kornfeld 1985). Synthesis and processing of *N*-linked glycoprotein oligosaccharides begins in the ER; processing continues in the Golgi complex by Golgi-resident glycosidases and glycosyltransferases that concentrate along the *cis* to *trans* axis following the order in which they act in the processing pathway. It is

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Abbreviations used: CHO-KI, Chinese hamster ovary; COP, coatomer protein; ct, cytoplasmic tail; ER, endoplasmic reticulum; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; GEM, glycosphingolipid-enriched domains; GFP, green fluorescent protein; GEM, glycolipid-enriched domains; GFP, green fluorescent protein; GGT, glycolipid glycosyltransferases; GlcT, glucosyltransferase; Ntd, N-terminal domain; TGN, trans-Golgi network; TMD, transmembrane domain..

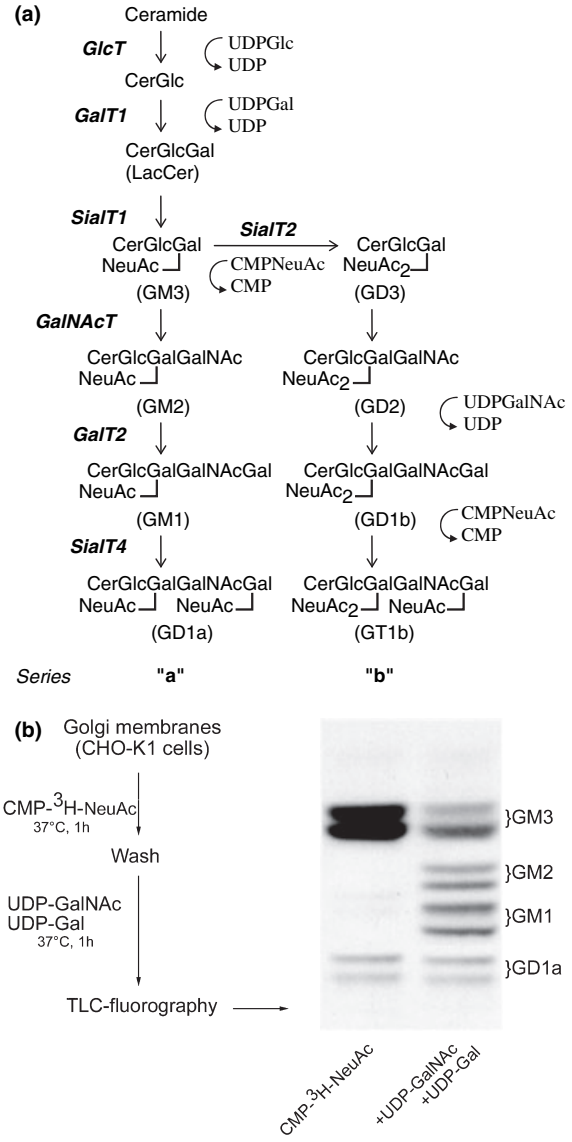

Fig. 1 (a) Simplified pathway of biosynthesis of gangliosides. Only the transfer steps for synthesis of "a" and "b" series gangliosides is shown. For a complete pathway and relevant references, see Maccioni *et al.* (1999) and Yu *et al.* (2004). (b) Functional coupling of transfer steps for conversion of LacCer to more complex gangliosides. As schematically shown at left, microsomal (Golgi) membranes of CHO-K1 cells expressing GalNAcT were incubated in a first step with CMP-^{3}H-NeuAc for 1 h and washed to eliminate unincorporated nucleotide. The washed membranes were incubated in a second step for 1 h without any addition or with non-radioactive UDP-GalNAc and UDP-Gal. The HPTLC-fluorography at right shows how radioactive GM3 formed in the first step was converted into GM2 and GM1 in the second step (modified from Giraudo *et al.*, 2001).

now accepted that there is considerable overlapping of these enzymes along sub-Golgi compartments (Rabouille *et al.* 1995). Unlike *N*-linked glycoprotein oligosaccharides, ceramide-linked oligosaccharides are not processed; rather, they are synthesized by glycosyltransferases that act in succession or compete at branching points for common-specific acceptors and do not necessarily require the ordered spatial disposition along the Golgi cisternae of glycoprotein oligosaccharide glycosyltransferases and glycosidases processing enzymes.

Lessons from studies with intact Golgi membranes *in vitro*

Studies of labeling of endogenous acceptors in intact Golgi membranes of neural retina (Maxzud *et al.* 1995) and Chinese hamster ovary (CHO-K1) (Giraudo *et al.* 2001) cells were most informative in showing the functional coupling of different transfer steps in glycolipid synthesis. This approach is based on the ability of Golgi membranes to incorporate sugars *in vitro* into intermediates in the pathway of completion upon incubation of Golgi vesicles with appropriate sugar nucleotide donors. It provides a picture of a dynamic process of synthesis carried out by membrane-bound enzymes, glycolipid acceptors, and sugar transporters, which is interrupted at the moment of membrane isolation and is resumed *in vitro* under controlled conditions.

The labeling showed that transferases acting at the transfer steps for synthesis of LacCer (GalT1), GM3 (SialT1), GD3 (SialT2), GM2 (GalNAcT), GM1 (GalT2), and GD1a (SialT4) (Fig. 1a) co-localize in isolated Golgi vesicles. Reactions are functionally coupled in the vesicles, as under experimental conditions that preclude vesicular coupling between compartments, the product of one transfer step is used for the next transfer step in the pathway if the cognate sugar nucleotide donor is provided (Maxzud *et al.* 1995). This was illustrated in Fig. 1b with an experiment using CHO-K1 cells membranes, showing that LacCer is converted into GM3 and this is used for synthesis of GM2 and GM1 if the necessary sugar nucleotides are provided. Radioactive GM1 is converted into GD1a if unlabeled CMP-NeuAc is also present (not shown).

Lessons from studies with brefeldin A *in vivo*

The results of Maxzud *et al.* (1995) needed to be interpreted in the light of previous results of studies with pharmacological agents that block intra Golgi transport such as brefeldin A (BFA) or impair distal Golgi function such as monensin. BFA inhibits the GTP/GDP exchange on the ADP-ribosylation factor (ARF), thus blocking the formation of coatomer protein I (COPI) vesicles. As a consequence, the *trans*-Golgi network (TGN) and *trans* most Golgi cisternae condense in a post-BFA compartment (Reaves and Banting 1992), while the proximal Golgi fuses with the ER membranes (Lippincott-Schwartz *et al.* 1989, 1991). Monensin is a carboxylic ionophore that causes a higher inhibition of the synthesis of complex gangliosides respect to that of simple ones (Maccioni *et al.* 1999). It catalyzes cation-for-proton exchange across membranes, and impairment of intra-Golgi membrane traffic seems to be due to the swelling of Golgi cisternae containing H^+-ATPases, which in the

presence of the ionophore accumulate osmotically active ions. These proton pumping activities concentrate in the *trans*-Golgi and the TGN (for review, see Mollenhauer *et al.* 1990), which would explain why biosynthetic steps of *cis*- and *medial*-Golgi are less affected by monensin than those of the *trans*-Golgi and TGN.

Studies with BFA indicate that the synthesis of LacCer and the simple gangliosides GM3, GD3, and GT3 occurs in a compartment more proximal in the Golgi than the one in which the complex gangliosides GM2, GM1, and GD1a is carried out (van Echten *et al.* 1990; Young *et al.* 1990; Rosales Fritz and Maccioni 1995; for a review, see Maccioni *et al.* 1999, 2002). Metabolic radioactive labeling of gangliosides in the presence of BFA causes accumulation of radioactive GM3, GD3, and GT3, with minor differences in total incorporation. Rosales Fritz and Maccioni (1995) noticed that in BFA-treated retina cells the metabolic labeling of gangliosides is higher than in untreated cells and that the accumulated GM3, GD3, and GT3 do not exit to the cell surface; so, although these experiments indicate that synthesis of these gangliosides occurs in the pre BFA compartment, due to the prolonged time of residence of the acceptor intermediates in this compartment they do not inform about how much of the total synthesizing enzyme resides there.

Lessons from biochemical and immunocytochemical studies

Maxzud *et al.* (1995) concluded that the common compartment supporting coupled conversion of LacCer to GD1a is distally located in the Golgi (TGN), and reasoned that GalT1, SialT1, and SialT2, and their corresponding acceptors are present in proximal Golgi compartments but extend their presence to the TGN; conversely, the TGN-concentrated GalNAcT was virtually absent or not functional in proximal Golgi compartments. Later on, determination of enzyme activities in subfractionated Golgi complex membranes using truncated analogs of ceramide and glucosylceramide as acceptors showed the highest activities of all the enzymes catalyzing the conversion of LacCer to complex gangliosides subfractionating with late Golgi compartment markers of liver cells (Lannert *et al.* 1998). Direct immunocytochemical examination of the localization of a *c-myc*-tagged form of GalNAcT transfected to CHO-K1 cells evidenced that this is a TGN located enzyme and that the mechanism underlying this localization, when saturated, leads to its mislocalization to proximal Golgi compartments (Giraudo *et al.* 1999). This was not so for the case of SialT2, as even using inducible promoters to keep the level of its expression at a minimum, it was mostly localized in elements of the proximal (*cis, medial,* or *trans*) Golgi and also functionally present in the TGN of CHO-K1 cells (Daniotti *et al.* 2000). Also, the transfer step for LacCer to GM3 conversion was found concentrated in a distal Golgi compartment of CHO cells (Allende *et al.* 2000).

Common acceptors compete at branching points of the pathway

Maxzud *et al.* (1995) clearly showed that the fraction of SialT1 and SialT2 extending to the TGN competes with GalNAcT for the utilization of common acceptors at branching points: LacCer was transformed into GM3 and GD3 if Golgi vesicles were incubated with CMP-NeuAc or increasingly into GM2 and GD2 if increasing amounts of UDP-GalNAc were added to the system. These results evidenced that at branching points (Fig. 1a) the balance between the activities of the competing enzymes rather than a compartment-based order or encounters influences whether i.e. GM3 is used for synthesis of "a" and "b" (or "c") pathway gangliosides. The activity balance could be determined either by the amount of enzyme or/and to variations in the lumenal environment affecting i.e. the fractional saturation with the respective sugar nucleotide donors or with the activating divalent cation, or even modifying the intralumenal pH.

The influence of the relative saturation with the sugar nucleotide donor was a concurrent factor contributing to the control of glycolipid expression in the developing rat retina. Most CNS structures of birds and mammals shift the expression of gangliosides from GD3 at early- to GD1a at late-developmental stages, which correlates with the up and down transcriptional regulation of, respectively, GalNAcT and SialT2 during development (Panzetta *et al.* 1980; Yu *et al.* 1988). However, the adult rat retina is characterized by expressing a low proportion of ganglio-series gangliosides relative to the expression of GD3 (Daniotti *et al.* 1991). This particular pattern of expression do not only correlates with the maintenance in the adult stage of an activity of SialT2 higher than that of GalNAcT and GalT2, but also with the presence of a developmentally up-regulated, Golgi-concentrated UDP-sugar pyrophosphatase activity (Martina *et al.* 1995; Martina and Maccioni 1996). This activity limits the availability of UDP-GalNAc and UDP-Gal, but not of CMP-NeuAc to the Golgi lumen, which contributes to direct the flow of LacCer towards formation of GM3 and GD3 instead of to a-series gangliosides. This seems to be a representative case of concurrent genetic and epigenetic factors that by lowering the activity of an enzyme that consumes an intermediate results in an elevated expression of that intermediate, as advanced by the multienzyme kinetic analysis for prediction of the steady state concentration of gangliosides (Bieberich and Yu 1999; Yu *et al.* 2004).

GGTs associate physically

Although the idea that glycosyltransferases may act as multienzyme complexes in the synthesis of glycans was considered many years ago (Roseman 1970; Caputto *et al.* 1974), the structural organization underlying this concept was investigated experimentally quite recently for the first

time for the GGT GalNAcT (GA2/GM2/GD2 synthase) and GalT2 (GA1/GM1/GD1b synthase) by Giraudo *et al.* (2001). They demonstrated that epitope tagged GalNAcT and GalT2 heterologously expressed in CHO-K1 cells mutually co-immunoprecipitate and that the immunocomplexes are able to couple the two transfer steps leading to the synthesis of GM1 from exogenous GM3. The Ntds of the transferases participate of the physical interaction, as shown by competition experiments and by determination of molecular proximity in living cells by fluorescence resonance energy transfer (FRET). FRET was positive in the Golgi area for the Ntds of GalNAcT and GalT2 fused to appropriate spectral variants of the green fluorescent protein (GFP), but not for the pair GalNAcT/ManII. ManII is another Golgi resident but of the glycoprotein processing pathway suggesting that some pathway specificity may underlie these interactions. Apart from improving the efficiency of glycolipid synthesis by channeling the intermediates from the position of product to the position of acceptor along the transfer steps, this is a clear case of kin oligomerization that may participate in the mechanism conferring Golgi residence to these proteins, as was suggested for the glycoprotein processing enzymes GlcNAcT1 and mannosidase II (Nilsson *et al.* 1996). Another important conclusion emanating from the use of the Ntds is that translocation in the ER, concentration at ER exiting sites, transport from the ER to the Golgi, concentration therein, and physical associations between some of them is codified in these quite few (about 50–60) amino acids.

Single versus various complexes

We also examined if other enzymes of the pathway were involved in complex formation. GalT1, SialT1, and ST2 were also found physically associated, in a SialT1 dependent manner, with participation of the Ntds; co-immunoprecipitation and fluorescence recovery after photobleaching (FRAP) experiments showed that these transferases interact poorly with GalNAcT and GalT2, suggesting that ganglioside synthesis is organized in distinct units formed by the association of particular GGTs. Triple color imaging of Golgi-derived microvesicles of nocodazole-treated cells showed dissociation of GalNAcT (and GalT2) from GalT1, SialT1, and SialT2, in a temporal pattern compatible with a more distal concentration of these enzymes (Giraudo and Maccioni 2003a). In F-11A cells, a substrain of neuroblastoma F-11 cells, SialT2 was found physically interacting with GalNAcT and with pyrene-labeled GM3, but not with SialT1 (Bieberich *et al.* 2002; Yu *et al.* 2004). These results seem at first sight at variance with those in CHO-K1 cells. However, as most higher eukaryotic cells express at least GM3 at the cell surface, and higher order gangliosides appear i.e. during differentiation, it is possible that a GalT1 and SialT1 basic complex is present in the majority of cells and that either other complexes are formed (as may be the case of CHO-K1 cells) or the basic complex adjust its complexity (as may be the case of F-11A cells) according to the differentiation dependent, up- or down-regulation of the participating transferases.

It is interesting to note that co-expression of interacting GGTs promoted their mutual activation, as it was the case of SialT2 and GalNAcT in F-11A cells (Bieberich *et al.* 2002) and of SialT2 and GalT1 and SialT1 in SialT2 transfected CHO-K1 cells (Uliana *et al.* 2006a). Also, it was reported that the recombinant soluble C-terminal catalytic domains of the β1,3GlcNAcT8 and β1,3GlcNAcT2 participating in the elongation of multiatennary *N*-glycans are able to associate and that the association enhances their enzymatic activities (Seko and Yamashita 2005). It is not known whether these activation events are of genomic or epigenomic nature.

The possibility that GGT associations involve segregation in specialized membrane domains of the Golgi complex, similar to the glycosphingolipid-enriched domains (GEM) at the cell surface (Simons and van Meer 1988; Simons and Ikonen 1997; Prinetti *et al.* 1999) was investigated in CHO-K1 cells by Crespo *et al.* (2004). They found that while plasma membrane GM3 and most GD3 and GT3 behave as GEM constituents in standard protocols for their characterization, their newly synthesized counterparts still in the Golgi do not. This suggests that glycosphingolipid products enter GEM after their synthesis in the Golgi cisternae, along the secretory pathway and/or at the cell surface. Interestingly, most of the GGT complexes in Golgi membranes do not behave as GEM constituents either. Triton X-100 insoluble complexes of interacting GlcNAcTI and GlcNAcTII through their lumenal domains have also been described in CHO cells (Opat *et al.* 2000), but these complexes do not fit into the classical "raft" characteristics as they were not affected by cholesterol depletion.

The transmembrane domains are relevant to the associations

As mentioned above, when both tail deleted or tail mutated Ntd of GalT2 and GalNAcT were co-expressed, they both stay in the ER as expected, but in an association close enough so as to undergo FRET (Giraudo and Maccioni 2003a). This indicates that complex formation between these two enzymes occurs in the ER, and also importantly, that complex formation depends more on the transmembrane domain (TMD) than on the cytoplasmic tail (ct). In line with this observation, co-transfection of tail deleted GalT2 with normal GalNAcT leads to the movement of an important fraction of tail deleted GalT2 towards de Golgi complex where it undergoes FRET with GalNAcT. FRAP experiments indicate that the rate of transport is essentially the same for GalT2/GalNAcT pairs in which both members of the pair have normal ct than for pairs in which only one member bear a proper ct (Giraudo and Maccioni 2003a). This indicates that for moving a complex from the ER to the Golgi, it is

sufficient that one of the members of the complex interacts with the export system to move the whole complex to the Golgi. So, complex formation complicates the interpretation of results of experiments in which the localization of a given GGT is evaluated after parts of it have been modified or deleted, as the association to endogenous partners may passively influence the localization of the mutated version. Disulfide bonded homodimers of GGT also occur in the ER; their relationships with enzyme activity and complex formation may vary for each protein and is an unsettled issue at this moment (for review, see Young 2004).

Other glycosyltransferase complexes

Complexes between glycosyltransferases acting on other glycosylation pathways have been described, which seem to assemble in the ER and exert their function in the Golgi apparatus. Examples are the uronosyl 5-epimerase/2-*o*-sulfotransferase complex (Pinhal *et al.* 2001) and the glycosyltransferase heterocomplex EXT1/EXT2 involved in heparan sulfate synthesis (McCormick *et al.* 2000) which are involved in the synthesis and co-polymerization of glucuronic acid and *N*-acetylgalactosamine during heparin sulfate biosynthesis, respectively. In yeast, two different types of Golgi mannosyltransferase complexes have been described (Jungmann and Munro 1998; Jungmann *et al.* 1999), which actively recycle through the ER (Todorow *et al.* 2000), a behavior shared by ganglioside glycosyltransferase complexes (Giraudo and Maccioni 2003b). Moreover, it has been proposed that these mannosyltransferase complexes regulate the expressed glycan diversity by altering their composition in mannan backbone (Stolz and Munro 2002). Conversely to the case of the EXT1/EXT2 complex that upon formation shifts from the ER to the Golgi (McCormick *et al.* 2000), the association of the ER located UDP-galactose:ceramide galactosyltransferase with the Golgi UDP-galactose transporter promotes a shift of the transporter towards the ER (Sprong *et al.* 2003). However, this is not the case for the Golgi located CMP-NeuAc transporter and the sialyl transferases, as redistribution of ST6Gal I STtyr isoform or ST8Sia IV polysialyltransferase to the ER is not accompanied by the redistribution of the Golgi CMP-NeuAc transporter (Zhao *et al.* 2006). Two glucuronyltransferases (GlcAT-P and GlcAT-S) and a sulfotransferase (HNK-1ST) involved in the human natural killer carbohydrate epitope (HNK) biosynthesis were found physically and functionally associated in the Golgi through their C-terminal catalytic domains (Kizuka *et al.* 2006). The organization of the assembly line for synthesis of glycans in multienzyme complexes containing different sets of transferases and functionally coupled may constitute a supramolecular organization capable of finely tuning glycoconjugate expression in response to regulatory cues. This control level may superimpose to that exerted through the transcriptional control of the expression of individual glycosyltransferases.

ER exiting of GGT

Most Golgi glycosyltransferases are synthesized and co-translationally inserted in the ER membranes with Type II topology; translocation is stopped by a signal anchor sequence, the TMD, located in the N-terminal region (Paulson and Colley 1989). From the ER they are transported as integral membrane proteins towards the Golgi complex where they concentrate and cycle through the ER (Lippincott-Schwartz *et al.* 1989). An imbalance between the rates of anterograde and retrograde transport, favoring anterograde transport, determines their concentration as residents of the Golgi complex, which was calculated to be 90 : 10 at steady state (Rhee *et al.* 2005). The molecular basis of the folding, transport and retention in the organelle of GGT are only partially known issues at present.

Folding requirements

When examined, *N*-glycosylation sequons (Asp-X-Ser/Thr) of GGT have been found occupied with *N*-linked oligosaccharides. *N*-linked oligosaccharides in GalNAcT (Haraguchi *et al.* 1995), GalT2 (Martina *et al.* 2000), and SialT2 (Martina *et al.* 1998; Daniotti *et al.* 2000) were found critical for the activity and subcellular localization of these transferases probably reflecting known oligosaccharide-mediated quality controls of folding (Trombetta and Parodi 2005). Inhibition of *N*-glycosylation of the only *N*-glycosylation site (Asn143) of GalT2 either by tunicamycin or by site-directed mutagenesis results in the loss of enzyme activity and retention in the ER. Castanospermine (an inhibitor of ER glucosidase I and II) partially impairs the exiting from the ER of a GalT2 with reduced K_m and V_{max} values for the substrates (Martina *et al.* 2000). All three *N*-glycosylation sequons of SialT2 were found glycosylated and necessary to attain and to maintain a catalytically folding, and for exiting the ER; *N*-glycan trimming in the ER, while not required for enzyme activity, is necessary for proper trafficking of SialT2 to the Golgi (Martina *et al.* 1998). In F-11 cells, ER retention and a fourfold increase in the turnover of SialT2, but not of GalNAcT, was observed upon castanospermine inhibition of *N*-glycan processing, which lead the authors to link processing and transport of SialT2 with its function in ganglioside biosynthesis (Bieberich *et al.* 2000; Yu *et al.* 2004).

The role of the ct-located [R/K](X)[R/K] motif

Proteins synthesized on ER membranes move along the secretory pathway as cargo in vesicles that bud in the form of COPII coated vesicles at exiting sites of the ribosome free, transitional ER membrane subdomains. The budding mechanism is accompanied by a selective concentration of the cargo as vesicles form (for reviews, see Bonifacino and Glick 2004; Wickner and Schekman 2005). The events underlying the selective concentration of cargo in these vesicles have

been studied using model proteins (i.e. the G protein of the vesicular stomatitis virus, a Type I membrane protein). These studies lead to the characterization in the proteins of diacidic signals (DxE) or dihydrofobic signals near the C-terminus of the protein that by interaction with the small GTPase Sar1 in a Sec23/24 dependent form, selectively concentrates them at these ER exiting sites. Signals for the ER exiting of Type II membrane proteins have been characterized within the long ct of soluble N-ethylmaleimide-sensitive attachment protein receptor (SNARE) proteins (for review, see Mancias and Goldberg 2005). However, these signals are not present in the relatively short ct of GGT, which lead us to examine the events supporting their ER exiting.

We carried out a series of experiments of deletions and/or mutation in the ct of the Ntds of GalNAcT, GalT2, and SialT2 fused to spectral variants of the GFP and examined the effects on the Golgi localization of the constructs (Giraudo and Maccioni 2003b). Tail deletions lead to a failure of these proteins to concentrate in the Golgi complex, remaining associated to membranes of the ER. Mutational analysis revealed a dibasic motif [R/K](X)[R/K] in the ct, proximal to the TMD, conserved among different organisms and uncommon in ER-resident Type II membrane proteins, as necessary for the concentration of the fusion proteins in the Golgi. FRAP experiments showed that the rate of ER to Golgi transport of a GalT2 construct having RR®AA replacement in the ct was about 30-fold lower than that of the normal construct. Transplantation of the ct of GalT2 to the ER resident, Type II membrane protein Iip33 (Schutze *et al.* 1994) promoted the exiting of the $GalT2_{ct}$-Iip33 chimera from the ER; this was not so if the ct RR®AA mutated version was used, confirming the ability of the [R/K](X)[R/K] motif to act as an ER export signal. Interestingly, the $GalT2_{ct}$-Iip33 chimera was found partially co-localizing with Golgi markers, suggesting that the ct may also have some capacity to drive Golgi concentration of the appended membrane-bound domain (see below).

In a search for binding partners of the [R/K](X)[R/K] motif, it was found that microsomal membranes of cells expressing GalT2, or synthetic peptides with the ct sequence of GalT2 bound to Sepharose bind recombinant Sar1, the small GTPase that initiate the formation of COPII coats at ER exiting sites. Binding of Sar1 was either in its GDP or GTP restricted form and on a [R/K](X)[R/K] motif-dependent manner. Sec23, a component of the first layer of the COPII coat, binds to Sar1/GalT2 in membranes and also to $Sar1_{(GTP)}$ bound to Sepharose-ct peptides. From these studies, we proposed the consensus sequence [R/K](X)[R/K], proximal to the TMD as necessary for ER exiting of these glycosyltransferases (Giraudo and Maccioni 2003b). The consensus sequence operates also in plant cells, as it was described for the ER exiting of the ER/Golgi membrane-bound prolyl hydroxylase of *Nicotiana tabacum* and *Arabidopsis thaliana.* (Yuasa *et al.* 2005) and Golgi α-mannosidase II of *A. thaliana* (Strasser *et al.* 2006).

Determinants for Golgi and sub-Golgi localization

The reason why GGT exiting the ER do not continue their trip along the secretory pathway to, for instance, the plasma membrane, is still an enigma. Several years ago, it was hypothesized that glycosyltransferases are retained in the Golgi complex because they cannot enter the small sized vesicles that leave the organelle either towards other elements of the exocytic pathway (i.e. endosomes and lysosomes) or to the plasma membrane. It was suggested that this may occur because a kin recognition of the transferases leads to formation of macromolecular complexes too large to accommodate in these vesicles (Fullekrug and Nilsson 1998). Within the same concept, it was proposed that exclusion was due to a hydrophobic mismatch between the width of the membrane of the transport vesicle (increased due to the increase in cholesterol content of flowing membranes) and the length of the TMDs of glycosyltransferases, which was calculated to be slightly shorter than the corresponding segment in plasma membrane integral proteins (Munro 1998). Although the arguments appear different, at this moment, one possibility do not necessarily exclude the other; however, the concept of size exclusion due to formation of macromolecular complexes seems to gaining in acceptance (for review see Graffenried and Bertozzi 2004).

As mentioned before, the Ntds are sufficient to convey reporter proteins or antigenic epitopes to the Golgi complex (see Colley *et al.* 1992; Fenteany and Colley 2005 and Uliana *et al.* 2006a). However, the question of why some transferases concentrate in the distal Golgi, while others spread more along proximal and distal Golgi elements has been difficult to address, in part due to methodological limitations. Few antibodies against the endogenous enzymes are available, which forced the heterologous expression of antigenic epitope- or GFP-tagged constructs for direct visualization by fluorescence and/or immunoelectron microscopy. In addition to the inherent limitations of the heterologous (over) expression of proteins that may saturate the mechanisms determining the fine subcellular localization, it is hard to distinguish the different sub-Golgi compartments under normal conditions. This last limitation prompted the use of BFA as a tool that, as mentioned above, dissects the Golgi complex *in vivo* into proximal (*cis, medial,* and *trans*) and distal (TGN) subcompartments.

Relevance of the ct(s)

For the case of GGT, Uliana *et al.* (2006a) studied the behavior of the Ntd of two enzymes, GalNAcT as a typical distal Golgi-resident enzyme (Giraudo *et al.* 1999) and SialT2 (Daniotti *et al.* 2000) as a representative of those spreading along the

proximal and distal Golgi. Single cell examination of CHO-K1 cells that co-express fusions of the Ntds of SialT2 and GalNAcT to appropriate spectral variants of the GFP in the presence of BFA showed preferential redistribution of the SialT2 into the ER, in comparison with that of GalNAcT that mostly remains concentrated in the post-BFA compartment. Subcellular fractionation of membranes from these cells confirmed the trends observed in dual color, single cell experiments. These results indicate that the Ntds of these enzymes carry information for such localization.

Within the Ntds, determinants for the particular sub-Golgi localization of these two enzymes reside in their ct, as swapping them between SialT2 and GalNAcT results in localization trends similar to those of the donors of these tails. The lack of homology in ct sequences (apart from the conserved ER exiting [RK(X)RK] motif described above), makes it less probable that a conserved-binding motif participates in the differential sub-Golgi concentration. Among several possibilities, Uliana *et al.* (2006a) considered that SialT2 may be actively retained in proximal Golgi elements, i.e. by interaction of its ct (MSPCGRARRQTSR-GAMA–VLAWKFPRTRLPM) with other cytoplasmically exposed Golgi protein partners. GalNAcT, with a shorter ct (MWLGRRA) would bind with less affinity to these partners or interact with different, more distally located ones. GalT1 and SialT1, which in CHO-K1 cells associate with SialT2 (Giraudo and Maccioni 2003a) could passively follow the behavior and dynamics of SialT2.

The ct of the Ntd of two *trans*-Golgi glycosyltransferases, β1,4GalT and α2,6 SialT were also reported to influence their respective localization in HepG2 cells; in monensin-treated cells, β1,4GalT dissociates from α2,6 SialT and concentrates preferentially in swollen, TGN-derived vesicles. Swapping their ct results in α2,6 SialT concentrated in the swollen vesicles, indicating that the ct of β1,4GalT is relevant to the response to monensin. Within the tail, the first 13 amino acids present in the long variant (resulting from translation initiation using the first of two in-frame methionines) were relevant to the monensin response (Schaub *et al.* 2006).

Milland *et al.* (2002) over-expressed a construct with the ct of the α1,3GalT fused to the sequence of the plasma membrane protein Ly9 , which resulted in the inhibition of the endogenous α1,3GalT activity and diminution of the surface expression of its product, which was interpreted as mislocalization of the enzyme by inhibition of its binding to specific sites in the Golgi. Unfortunately, no conclusive evidence of mislocalization of α1,3GalT was provided in this interesting study. It is still possible that ER retention of α1,3GalT due to inhibition of its ER exiting by the over-expressed ct-Lys9 construct (a dominant negative effect for binding to COPII components) was followed by ER associated degradation (Tsai *et al.* 2002) of the retained α1,GalT as it was shown for human core 1β3-GalT (Ju and Cummings 2002). It is expected that future work will reveal how the information for sub-Golgi localization in the Ntds is interpreted by the organelle in molecular terms. Within the frame of the cisternal maturation (Fullekrug and Nilsson 1998; Glick and Malhotra 1998) or of vesicular transport (Rothman 2002) models of Golgi complex dynamics, the possibility of interactions of the ct(s) of GGT with coat or intercisternal proteins for, respectively, COPI coat interactions or retention, emerges as plausible and interesting.

Plasticity of fine sub-Golgi localization

An interesting question emerging from the organization in complexes of GGT is the one referred to how the topological distribution of the complexes in Golgi subcompartments is affected when a partner of the association is missing or over-expressed. Uliana *et al.* (2006b) addressed this issue in CHO-K1 cells, which express the transferases necessary for the synthesis of GM3 (GalT1 and SialT1), but lack SialT2, another member of the GalT1/SialT1/SialT2 complex (Giraudo and Maccioni 2003a). They found that CHO-K1 cells stably expressing SialT2 (SialT2$^+$ cells) show a fraction of LacCer uncoupled from synthesis of higher order glycolipids, indicating a change in the functional relationships of transfer steps underlying LacCer formation and utilization induced by SialT2; the pattern of glycolipid synthesis in the presence of BFA or monensin suggested that transfer steps acting beyond GlcCer localize in sub-Golgi compartments more distally located in SialT2$^+$ cells that in the wild-type cells. The "distalization" was evidenced by subcellular fractionation and by single cell imaging experiments with cells co-expressing the Ntds of GalT1and SialT1 fused to appropriate spectral variants of the GFP in the presence of BFA. The fraction of the chimeras localized in a post-BFA compartment, partially co-localizing with markers of the TGN and recycling endosomes was higher in SialT2$^+$ cells than in wild-type cells. These results were compatible with SialT2 expression promoting a "distalization" of the complex GalT1/SialT1/SialT2, although the molecular mechanism it is not known at present.

As mentioned before, glycosyltransferase coding genes are under transcriptional control, and the promoter sequences controlling their expression have been characterized (for a review, see Yu *et al.* 2004). However, less is known at present about how the complex organization of the machinery for synthesis in the Golgi accompanies the regulatory fluctuations in the input of participating enzymes. This occurs in many biological circumstances, as for example during CNS development, in which up and down transcriptional regulation of GalNAcT and SialT2 occurs (reviewed in Maccioni *et al.* 1999 and Yu *et al.* 2004). Recently, it was reported that increased expression of the Golgi enzyme GalNAcT2 induced increased COPII assembly at ER exit sites via a direct interaction with Sar1p and also lead to an

overall increase in the size of the Golgi apparatus (Guo and Linstedt 2006). Taken together, these results and those mentioned before of the redistribution of the UDP-Gal transporter to the ER when GalCer transferase is over-expressed (Sprong *et al.* 2003) or of Mn9p from the ER to the Golgi (Todorow *et al.* 2000) are probably just the beginning of examples of adaptive responses of the Golgi complex to fluctuations in the relative proportion of its components.

Perspectives

In the last 15 years, molecular cloning of GGT allowed us to progress not only in the understanding of the biological role of glycolipids (Proia 2004) but also to approach the cellular and molecular basis of the organization of their synthesis in the Golgi complex. Molecular dissection of GGT has been carried out in different laboratories in the search for critical domains for exiting the ER, for concentration in Golgi and sub-Golgi compartments, for their mutual functional relationships, etc. We are less ignorant about these issues, but we are still at a stage in which information coming from different cell and expression systems, different pathways of glycosylation, different tagging strategies (to circumvent the lack of specific antibodies against the endogenous enzymes) accumulates in a turbulent fashion. The knowledge of the cellular basis of the organization of glycolipid synthesis in the Golgi complex is shedding light on important aspects of the functional organization of the organelle. Emerging evidence shows the Golgi complex as a supramolecular ensemble with capacity of remodeling the topology of the synthesizing machinery to accompany regulatory responses of the cell involving changes of cell surface glycolipid composition. It is expected that the combination of modern imaging technologies in living cells with the tools of cellular and molecular biology we are now applying will soon make clearer the somehow cloud-capped picture of the synthesis of glycolipids that we have at present.

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