The roles of enzyme localisation and complex formation in glycan assembly within the Golgi apparatus
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Cell surface glycans govern numerous cell–cell interactions and are therefore key determinants of multicellular biology. They originate from biosynthetic pathways comprising an assembly line of glycosyltransferases within the Golgi compartment. Although the mechanisms of Golgi enzyme localisation are still under debate, the distribution of these enzymes among the Golgi cisternae can dictate the overall structures produced by the cell. Fine-tuning of glycan biosynthetic pathways is further accomplished by specific associations among glyclosyltransferases. Together, localisation and association govern the assembly of complex glycans and thereby regulate interactions at the cell surface.

Introduction
The Golgi apparatus is responsible for processing proteins and lipids that are biosynthesised in the endoplasmic reticulum (ER), post-translationally modifying them to modulate their stability and function, and then delivering them to different locations within and outside the cell. The Golgi apparatus comprises flattened cisternae with dilated rims; these cisternae are most often organised in the form of a stack [1]. This structure compartmentalises the modifying enzymes, allowing the sequential initiation and elaboration of post-translational modifications. Glycosylation, the most prevalent and complex form of post-translational modification, is accomplished by glycosyltransferases that are organised in an assembly line established by differential localisation among the Golgi cisternae. Although the mechanisms allowing the selective retention of glycosyltransferases in certain cisternae are still poorly understood, their relative positions are known to govern, in part, the structures of glycans produced by the cell [2]. Thus, enzyme localisation is an important determinant of the carbohydrate repertoire expressed on the cell surface. The recent discovery of Golgi enzyme clusters that direct the biosynthesis of specific glycan structures has shown that other mechanisms besides compartmentalisation may be necessary to ensure that certain glycoforms are generated. This review summarises current models of Golgi enzyme localisation and the role of enzyme association, which can affect localisation, in the genesis of glycan structures. These processes within the Golgi are vital to understanding how glycan-dependent cell–cell interactions are regulated.

Mechanisms of Golgi enzyme subcompartmentalisation
The mechanisms involved in localising proteins to the Golgi apparatus are contingent on the mode of transport through the organelle. Until recently, it was thought that Golgi enzymes are confined to cisternae and that cargo are trafficked through the Golgi in the anterograde direction via COPI vesicles [3]. According to this model, Golgi localisation would be an anchoring process involving exclusion from vesicles. The vesicular transport model has been challenged by the cisternal maturation model. In this scenario, cargoes remain in the cisternae, which are the main anterograde carriers, while the modifying enzymes are trafficked backward by COPI vesicles [4]. The cisternal maturation model predicts that COPI vesicles should be enriched for Golgi-resident enzymes but depleted for cargo, and that cargo should remain confined in a specific cisterna; both of these predictions have been observed experimentally [5–7]. While the evidence for and against cisternal maturation continues to be debated (see several excellent reviews published recently [8–10]) this article will discuss localisation of Golgi resident proteins in the context of the cisternal maturation model.
If we assume that the cisternal maturation model is valid, then Golgi localisation requires active partitioning into vesicles rather than an anchoring mechanism. Models based on a static Golgi suggest that proteins localise to the Golgi via two mechanisms: aggregation that limits packaging into vesicles, or selective partitioning of each protein into a cisterna where the thickness of the membrane is equivalent to the length of the transmembrane domain of the protein [11,12]. Aggregation of glycosyltransferases has been observed in the case of the medial Golgi enzyme N-acetylgalactosaminyltransferase I (GlcNAcTI); however, it still reaches the trans-Golgi network (TGN) at a rate similar to that of a cargo protein, arguing that these higher order structures are not impeding the enzyme’s movement [13,14]. The membrane thickness model is more consistent with a maturation view of the Golgi, but its premise has recently been questioned. The membranes of the secretory pathway are thought to increase in thickness en route to the plasma membrane as a result of increasing levels of cholesterol and sphingolipids. Thus, the cisternae of a specific compartment could only accommodate hydrophobic transmembrane domains of matching length [12,15]. Solution X-ray scattering experiments performed by Engleman and colleagues showed that removal of cholesterol from purified membranes ranging from the ER to the plasma membrane had limited effects on the thickness of the bilayers [16**]. However, treatment of the isolated membranes with proteases decreased the thickness of the ER bilayer and increased that of the Golgi, arguing that the proteins present in the membrane may force the surrounding lipids to compress or expand to match the length of their transmembrane domains. The idea that proteins embedded within the membrane determine its thickness, rather than the other way around, is difficult to reconcile with a gradual thickening of the bilayer functioning as a localisation mechanism. It is important to note that the X-ray measurements correspond to averages, and it is therefore possible that domains of varying thickness exist within each membrane. COPI vesicles bud from regions of cisternae composed of thinner membranes, suggesting that there is heterogeneity in the bilayer of the cisterna or that the machinery responsible for forming a vesicle can actively change the local composition of the membrane [17].

As proposed, the cisternal maturation process could produce gradients of enzymes in the Golgi as well as diffuse distributions that have been observed for several glycosyltransferases. For example, polypeptide N-acetylgalactosaminyltransferase I (ppGalNAcTI), one of several enzymes responsible for initiating O-linked glycan biosynthesis, is found throughout the Golgi, as assessed by immunoelectron microscopy [18]. A simple way to account for this type of localisation would be exclusion from COPI vesicles altogether. In this way, the enzyme would remain in an individual cisterna as it matures and then recycle back to a developing cisterna after reaching the TGN. Recycling would require a direct method of transport from the TGN to the early parts of the secretory pathway, a function that could be supplied by the specialised domains of the ER that have been shown to associate with the TGN [19]. These domains have been proposed to play a role in non-vesicular transport of ceramide [20] but this may not be their exclusive function. Inclusion or exclusion from vesicles may not be an absolute requirement for proper localisation. There may be several mechanisms for localising proteins to the Golgi that employ vesicular traffic, selective retention in maturing cisterna, or some combination thereof to ensure that the enzymes efficiently encounter and modify their substrates.

**Functional significance of Golgi enzyme associations**

In recent years it has become apparent that a variety of glycosyltransferases and related Golgi enzymes associate with one another as a means to control their activity. Glycosylated proteins typically show heterogeneity in their carbohydrate structures as a result of competition between different enzymes for the same substrate [21]. While it can be argued that this heterogeneity promotes functional diversity among molecules at the cell surface, there are cases where generating specific glycans is necessary. In these cases, the stochastic nature of glycan biosynthesis can be overcome by linking enzymes together to increase the probability that they will function in sequence. While dimerisation of glycosyltransferases, frequently via disulfide bonds, has been known for some time to affect Golgi localisation [22–24], we will confine our review to cases of heterodimerisation that are likely to influence biosynthetic outcomes.

**Complexes among N-linked glycan biosynthetic enzymes in mammals and yeast**

One of the first reported cases of association involves mannosidase II (MannII) and GlcNAcTI, enzymes that contribute to N-linked glycan biosynthesis [25]. The sequential action of these two enzymes is vital to the production of mature N-linked glycans [26]. The association of these enzymes was observed by grafting the cytoplasmic tail of the p33 invariant chain to GlcNAcTI, which retained it in the ER and forced MannII to localise to the ER as well. The regions of the enzymes involved in their interaction were probed by mutagenesis of various domains. The canonical glycosyltransferase architecture comprises an N-terminal cytosolic tail, a single-pass transmembrane domain, a luminal stem region and a C-terminal catalytic domain (Figure 1). The association of MannII and GlcNAcTI was shown to occur through the stem regions and can be disrupted without altering localisation, suggesting that association and localisation are controlled by different domains of the enzymes [27].
Figure 1

GlcNAcT1 and MannII associate via their stem regions. The two enzymes share the glycosyltransferase architecture comprising a cytosolic tail, single pass transmembrane domain (TMD), a stem region, and a globular catalytic domain. A charged region in the stem of GlcNAcT1 mediates the association of the enzymes, coupling the addition of GlcNAc to the N-linked glycan by GlcNAcT1 and subsequent trimming of mannose residues by MannII. The processed glycan is then elaborated by other glycosyltransferases to generate a mature N-linked structure.

Two distinct complexes of glycosyltransferases control the biosynthesis of N-linked glycans in *Saccharomyces cerevisiae*. Although the core glycan added to nascent polypeptides in the ER is similar in yeast and mammals, the pathways diverge dramatically upon reaching the Golgi. In *S. cerevisiae*, a single mannose residue is transferred by Och1p; this is followed by elaboration of an oligomannose glycan comprising α-1,6-and α-1,2-linked residues [28]. Three related genes, *ANP1*, *MNN9* and *VAN1*, all possessing homology to glycosyltransferases, were implicated in the synthesis of this polymer. Co-localisation experiments determined that the proteins are localised to the cis Golgi and subsequent immunoprecipitation showed that Mnn9p forms distinct complexes with both Anp1p and Van1p [29]. The Mnn9p–Van1p complex, named M-Pol I, was implicated in extending the single mannose residue by ~15 additional mannose residues. Both enzymes have α-1,6 mannosyltransferase activity and were necessary to initiate the polymer, but Mnn9p was shown to function as an α-1,2 transferase as well [30]. The Mnn9p–Anp1p complex, named M-Pol II, was shown to contain three other glycosyltransferases and to extend the polymannose structure initiated by M-Pol I, generating the full length glycan [31]. This complex showed limited α-1,2 mannosyltransferase activity and deletion experiments suggested that Mnn9p had limited α-1,6 activity, suggesting that its function varies between the two clusters. The presence of Mnn9p in both complexes is striking and suggests that a single enzyme may be able to catalyse different reactions depending on which partner it is associated with.

**Enzyme complexes in glycolipid biosynthetic pathways**

The association of Golgi enzymes plays an important role in glycolipid biosynthesis by shunting substrates along certain pathways to generate specifically glycosylated products. Several of the enzymes responsible for generating a subset of glycolipids known as gangliosides have been shown to associate. Coimmunoprecipitation and FRET experiments were used to show that N-acetylgalactosaminyltransferase (GalNAcT) and galactosyltransferase 2 (GalT2), the enzymes responsible for converting the glycolipid GM3 to GM1 (Figure 2), associate through their N-terminal domains [32]. Immunoprecipitated complexes of the two enzymes efficiently converted GM3 to GM1, which could not be duplicated by the mixture of lysates expressing each enzyme individually. This observation suggests that the interaction between these proteins is not spontaneous and may occur while the enzymes are folding. Cells co-expressing the two full-length enzymes and the N-terminal region of GalT2 showed diminished activity, indicating that the N-terminal domain competes with full-length GalT2 for binding to GalNAcT and that this interaction is necessary for efficient substrate processing.

An association has also been observed among three enzymes, galactosyltransferase 1 (GalT1), sialyltransferase 1 (SialT1), and sialyltransferase 2 (SialT2), that are involved in the biosynthesis of ganglioside precursors (Figure 2) [33**]. Whereas the enzyme pairs GalT1–SialT1 and SialT1–SialT2 co-immunoprecipitated efficiently, GalT1–SialT2 associated only in the presence of SialT1, suggesting that this enzyme is vital to forming the trimeric complex. The GalT1–SialT1–SialT2 complex resides further cis in the Golgi than the GalNAcT–GalT2 complex, which is consistent with the sequence of events in the ganglioside biosynthesis pathway. Another group has reported that SialT2 and GalNAcT associate in murine neuroblastoma cells, which contradicts the idea of two distinct biosynthetic clusters [34]. It is possible that different complexes of glycosyltransferases can form in different cell lines or that GalNAcT can form more than one cluster, as is the case with Mnn9p in yeast. The identification of two complexes involved in ganglioside production that are spatially separated in the Golgi shows that biosynthetic pathways can take advantage of both the cisternal structure of the Golgi and associative mechanisms to generate specifically glycosylated structures.

Association may also influence other classes of proteins that play important roles in glycan biosynthesis. Sugar
nucleotides, the activated sugar donors used by all glycosyltransferases, are pumped into the Golgi from the cytosol by membrane-bound transporters [35]. Although the distribution of these proteins in the secretory pathway is not well understood, the transporter for UDP–Gal, known as UGT, is confined to the Golgi apparatus and its activity is not found in the ER. Galactosylceramide (GalCer), a glycolipid precursor important in spermatogenesis and myelinating cells, is biosynthesised from ceramide and UDP–Gal by a galactosyltransferase found exclusively in the ER [36]. The question of how an ER-resident glycosyltransferase could function without a source of substrate was answered by showing that the enzyme selectively retains a fraction of UGT in the ER (Figure 3) [37**]. The retention appears to be specific, as a CMP–sialic acid transporter is not retained. Considering that the GalCer galactosyltransferase is expressed in limited cell types, global retention of UGT in the ER would misdirect UDP–Gal to a compartment where it would not be utilised. By coupling the expression of the GalCer galactosyltransferase to redistribution of UGT to the ER, a biosynthetic pathway can be established only when required.

Complexes among enzymes in glycosaminoglycan biosynthetic pathways

Association of glycosyltransferases involved in the production of glycosaminoglycans (GAGs) has also been observed. Heparan sulfate is a GAG whose biosynthesis begins with the assembly of a polymer composed of repeating units of glucuronic acid (GlcA) and N-acetylgalactosamine (GlcNAc). This polymer is then epimerised, converting GlcA to iduronic acid (IdoA), and heavily sulfated [38]. The underlying polymer is generated by the action of two proteins known as EXT1 and EXT2 (Figure 4a). Both proteins possess GlcA transferase (GlcAT) and GlcNAcT activity in vitro, suggesting that they might be functionally redundant [39]. However, a cell line deficient in EXT1 but still expressing EXT2 did not generate heparan sulfate, indicating that EXT2 was not capable of producing the GAG scaffold by itself. Each enzyme expressed separately was found localised in the ER, inconsistent with previous work suggesting that heparan sulfate chain extension occurs inside the Golgi [40]. Remarkably, when coexpressed, the two enzymes localised in the Golgi and could be coimmunoprecipitated. Lysates from cotransfected cells showed dramatically enhanced GlcNAcT and GlcAT activity compared to the single transfectants, implying that the associated enzymes function collectively as the physiologically relevant heparan polymerase. Deletion of 300 amino acids from the C-terminus of EXT1 caused the truncated EXT1, along with EXT2, to localise in the ER, which the authors attributed to a loss of association. However, a lack of association was not directly demonstrated. It is possible that the truncated EXT1 does not fold properly and is prevented from exiting the ER, thereby retaining EXT2 in that compartment.

Epimerisation (inversion of a stereocenter) of GlcA to IdoA during heparan sulfate biosynthesis, a process catalysed by GlcA epimerase, is frequently followed by sulfation of the 2-position of IdoA by IdoA 2-O-sulfotransferase (2OST) to prevent reversion to GlcA [38] (Figure 4b). Like most Golgi-resident sulfotransferases, 2OST has a similar predicted architecture to the majority of glycosyltransferases. Under normal circumstances, both GlcA epimerase and 2OST are present in the medial Golgi. When 2OST was tagged with the p33 invariant chain, which retained it in the ER, the GlcA epimerase was found to be localised to the ER as well, suggesting that the enzymes form a complex [41]. In cells deficient in 2OST, the GlcA epimerase was present exclusively in the ER, showing that the epimerase requires the sulfotransferase to localise correctly. The enzymes coimmunoprecipitated and were only extracted from Golgi membranes in the presence of high salt, which is consistent with their in vivo association. The epimerase activity of 2OST-deficient cells was restored upon transfection with the sulfotransferase, implying that complex formation is necessary for the localisation and activity of
the epimerase. The lack of an epimerase-deficient cell line has made it difficult to determine if 2OST requires the epimerase to localise correctly, as observed with EXT1 and EXT2, or if 2OST is the dominant figure in this association.

**O-Linked glycan biosynthesis**

While association between different enzymes in the Golgi has been observed in a variety of glycosylation pathways, such observations are conspicuously absent in studies of O-linked glycosylation. While it is possible that interactions have yet to be discovered, it is interesting to note that differences in how O-linked glycans are biosynthesised may mean that association is a less viable strategy. Considering that O-linked structures can be initiated throughout the Golgi by up to 24 different ppGalNAcTs in humans, it is unlikely that one discrete complex of enzymes is responsible for generating early intermediates in this pathway [42]. The polypeptide substrate preferences of the GalNAcTs (which are poorly defined at present) may, along with knowledge of the enzyme’s localisation, enable us to determine the point at which the O-linked structure is initiated in the Golgi and what subsequent enzymes are capable of modifying it. A common elaboration of both N- and O-linked glycans is the N-acetyllactosamine (LacNAc, Galβ1,4GlcNAc) motif, often found in the form of repeating disaccharide units [43,44]. The GalTs and GlcNAcTs responsible for generating LacNAc repeats seem good candidates for association, but no evidence for this has been reported.

Novel mechanisms of regulation employed by the proteins RCAS1 and Cosmc may be important factors determining the structures of O-linked glycans [45**,46**]. Overexpression of the Golgi-resident protein RCAS1 has been shown to increase the levels of the Tn glycan, a truncated epitope associated with suppression of O-linked glycan extension enzymes. The precise function of RCAS1 is unknown but it may either downregulate certain glycosyltransferases or deny them access to their substrates. Cosmc is a chaperone within the secretory pathway that is required for the folding and activity of core 1 GalT, a key enzyme in O-linked glycan elaboration. Cells deficient in Cosmc lack core 1 GalT activity despite the presence of its transcript, leading to the production of truncated O-glycans.

**Future directions**

The interaction of enzymes within the Golgi apparatus has been confirmed in a variety of glycosylation pathways, but the molecular bases of the interactions and their relevance to the biosynthesis of glycans remain largely
undefined. Characterisation of the regions and specific amino acids responsible for the interactions should be a priority. At present, only the interaction between GlcNAcT1 and MannII has been mapped, in this case to a charged region in the stem region of GlcNAcT1 [27]. It will be interesting to see if different complexes associate through different domains or if a particular domain is always involved. More importantly, mapping the interacting domains will allow variants of the enzymes to be generated that no longer associate. Although it is assumed that association is important for channelling substrate to specific products, there are little data available to verify this hypothesis. In the case of GlcNAcT1, mutation of the charged residues in the stem had no effect on the enzyme’s activity when expressed in GlcNAcT1-deficient cells [14]. RNAi should allow the rapid identification of associating enzymes in cases where complex formation is necessary for correct localisation and could be used as a tool for screening for likely biosynthetic partners. Finally, engineered interactions between glycosyltransferases might be employed to elevate the production of a particular glycoform and may facilitate the production of homogenously glycosylated proteins.

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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
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Originally characterised as a cell surface protein involved in apoptosis, the authors show that RCAS1 is actually a Golgi resident protein and that its overexpression leads to the truncation of O-linked glycans.


The core 1 galactosyltransferase is vital for the production of most common O-glycans. The protein Cosmc functions as a molecular chaperone for this enzyme, thus controlling an important junction in O-linked biosynthesis. Jurkat cells have a mutation in Cosmc, leading to proteasome-mediated degradation of the galactosyltransferase, which can be prevented by complementation with the functional chaperone.