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Intact Golgi synthesize complex branched O-linked chains on glycoside primers: Evidence for the functional continuity of seven glycosyltransferases and three sugar nucleotide transporters

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We examined the functional co-localization and continuity of glycosyltransferases and sugar nucleotide transporters in the Golgi of two Chinese hamster ovary (CHO) cell lines that synthesize different types of O-linked oligosaccharides. CHO cells normally synthesize primarily Sia2,3Galβ1,3GalNAc- on glycoproteins. CHO cells transfected with core-2 GlcNAc transferase (Core 2) can synthesize glycoproteins containing branched O-linked oligosaccharides with poly-N-acetyllactosamines. CHO lines incubated with [^{3}H]galactose and GalNAc-α-phenyl (GAP) as a primer, synthesize labeled glycoside products that faithfully resemble those found on the endogenous acceptors: CHO cells make Sia2,3[^{3}H]Galβ1,3GAP, while CHO Core2 cells synthesize GAPs with complex branched chains including poly-N-acetyllactosamines. To determine if isolated Golgi preparations make similar products, we prepared Golgi by established homogenization methods, documented their intactness, and added tracer UDP-[^{3}H]Gal, unlabeled sugar nucleotides, and GAP. CHO Golgi preparations synthesized only Sia2,3[^{3}H]Galβ1,3GAP. CHO Core2, also made this product and a small amount of Core-2 GlcNAc transferase-dependent products. No endogenous glycoproteins were labeled. However, when either cell line was gently permeabilized with streptolysin-O or given hypo-osmotic shock, both GAP and endogenous acceptors were efficiently glycosylated within an intact functional Golgi lumen and remained there. Significantly, Golgi from CHO Core2 cells made mostly branched GAP products including some with poly-*N*-acetyllactosamines as complex as those made and secreted by living cells incubated with GAP. These results suggest that the lumen of the Golgi apparatus is functionally continuous or interconnected. Once glycosides diffuse into the Golgi lumen, they have access to all the sugar nucleotide transporters and glycosyltransferases used for complex GAP-based products without requiring metabolic energy or inter-vesicular transport. Glycosylation of artificial acceptors could be used to track the functional continuity or co-localization of multiple glycosyltransferases and transporters under conditions where Golgi morphology disintegrates and/or reappears.

Keywords: **Golgi, glycoside, glycosyltransferase, sugar nucleotide, CHO cell, streptolysin O**

Abbreviations: **CHO, Chinese hamster ovary cells; HPLC, high performance liquid chromatography; NDV, Newcastle disease virus; AUS, *Arthrobacter ureafaciens* sialidase; GAP and GalNAcαphenyl, phenyl-2-acetamido-2-deoxy-α-D-galactopyranoside; XylβMU, 4-methylumbelliferyl-β-xyloside; SE, supernatant extract; SP, supernatant pellet; GalTn, galactosyltransferase; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Sia, *N*-acetylneuraminic acid; PBS, phosphate-buffered saline; SLO, Streptolysin-O.**

Introduction

Artificial glycosides prime oligosaccharide biosynthesis within cells [1,2]. The best known examples are 4-methylumbelliferyl-β-xyloside (Xyl-β-MU) for glycosaminoglycan chains, and GalNAc-α phenyl (GAP) for O-linked chains [3]. In some cases, disaccharide primers such as Galβ1, 4GlcNAcβ-R have also been used [1]. The hydrophobic aglycones permit the

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glycosides to penetrate the plasma and Golgi membranes where they encounter multiple glycosyltransferases. The glycoside products secreted into the medium often faithfully resemble the repertoire of naturally occurring chains, and sometimes the primers selectively inhibit the synthesis of similar oligosaccharide chains on native endogenous acceptors.

Intact, correctly oriented purified Golgi preparations can also glycosylate primers [3–5]. Their glycosylation requires co-localization of sugar nucleotide donors and glycosyltransferases in the same Golgi lumen or compartment [3]. Specific transporters concentrate the donors within the Golgi lumen where transferases preferentially reside within the *cis*-, *medial*-, or *trans*-Golgi compartments or the *trans*-Golgi network [6].

We have used GAP as a freely permeable glycoside to study the functional organization and continuity of the Golgi in CHO cells transfected with core-2 GlcNAc transferase, which promotes synthesis of branched oligosaccharides with poly-*N*-acetyllactosamine chains [7]. If the Golgi is functionally divided into separate compartments containing non-overlapping sets of glycosyl transferases and sugar nucleotide transporters, only a subset of the possible structures will be formed. If the Golgi lumen is continuous or an interconnected network, GAP may be extended to structures that resemble those made by intact cells. When gentle cell lysis conditions are used to preserve Golgi integrity, our results suggest that the Golgi lumen is sufficiently interconnected or continuous to glycosylate GAP to the same extent as living cells.

Materials and methods

Materials and cells

Most of the materials were obtained from Sigma except for the following: α-MEM and geneticin disulfate from Gibco BRL; FBS from Hyclone Laboratories; L-glutamine, penicillin G and streptomycin sulfate from Irvine Scientific; trypsin-EDTA solution from ICN; C18 silica gel from Analtech Inc.; and Microsorb-MV NH_2 HPLC column from Varian Instruments. [6-^{3}H]galactose (60 Ci/mmol), UDP-[6-^{3}H]galactose (60 Ci/mmol), and UDP-*N*-acetyl-[6-^{3}H]glucosamine (60 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. Jack bean β-galactosidase, bovine testis β-galactosidase, *Arthrobacter ureafaciens* sialidase, Newcastle disease virus sialidase, jack bean β-*N*-acetylhexosaminidase, and *Streptococcus pneumoniae* β-*N*-acetylhexosaminidase were from Oxford GlycoSciences, which is now available from Glyko, Inc, Novato, CA. Ilimaquinone was a generous gift from Dr. Vivek Malhotra, University of California, San Diego, La Jolla, CA. CHO-leu was transfected with leukosialin and used as control line. CHO-leu cells additionally transfected with Core 2 GlcNAc-transferase are termed CHO·C2GnT and were kindly provided by Dr. Minoru Fukuda, The Burnham Institute [7]. Cell lines were cultured in α-MEM supplemented with 10% heat-inactivated FBS, 4 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, gentamicin sulfate and geneticin disulfate.

Labeling of cells with [^{3}H]Gal

Confluent CHO cells were washed twice with PBS and incubated in serum-free DMEM (0.1 mg/ml glucose) containing 250 μM GalNAc-α-phenyl (GAP). 10 μCi/ml [^{3}H]Gal was added to the medium and incubated at 37°C for 16 h. The medium was collected and applied to a 1 ml QAE-Sephadex column equilibrated in 2 mM Tris base and then washed with 3 ml of 150 mM NaCl. This step was essential to remove substances, which interfered with the subsequent fractionation of the GAP products. The run-through and 150 mM NaCl washes were applied to a C18 SepPak cartridge equilibrated with water and washed sequentially with 0.1 M ammonium formate, pH 6.0 and water. Glycoside products were eluted with 50% methanol and analyzed by HPLC as described below.

Isolation of Golgi-enriched subcellular fraction

Golgi-enriched membranes were prepared from CHO or CHO·C2GnT as described previously [3]. Purification of Golgi membranes was monitored by enrichment of galactosyltransferase (UDP-Gal:GlcNAc Galβ1,4-galactosyltransferase) as described previously [8]. Enrichment was typically 6–10 fold with 10–16% recovery.

Incubation of Golgi fraction with UDP-[^{3}H]Gal

Portions (0.5 mg) of the Golgi-enriched fraction were incubated with 1 μM UDP-[^{3}H]Gal (2.5 μCi; 10 Ci/mmol) and varying concentrations of GAP as described by Etchison and Freeze [3]. Incubations were done at room temperature (22–23°C) for 15 min and were chased for an additional 15 min following addition of a mixture of unlabeled sugar nucleotides containing 220 μM each of UDP-Gal, UDP-GlcNAc, and CMP-Sia in 5 mM $MgCl_2$ in 50 mM sodium maleate buffer, pH 6.5, (MGM) to give a final concentration of 5 μM each.

To verify that the incubations with the Golgi-enriched fraction require the transport of nucleotide sugars, the reactions were carried out as above in the presence of 0.2% Triton X-100. This disrupts the Golgi membranes and prevents the transporters from concentrating donor substrates within the Golgi lumen. To demonstrate the absence of intercompartmental transport, reactions were carried out as above after pretreatment of the Golgi-enriched fraction with 2 mM *N*-ethylmaleimide [9], or 30 μM ilimaquinone [10], or co-incubation with GTPγS. All of these agents inhibit vesicular transport. In addition, hexokinase, and glucose were added to deplete any traces of ATP. To verify that the purified Golgi vesicle fraction was properly oriented and sealed, protease protection of radiolabeled endogenous acceptors was assayed using the methods described previously [8].

Streptolysin-O (SLO) permeabilization

SLO permeabilization was carried out as described previously by [11] with modifications. CHO cells in a T-75 tissue culture flask were harvested by trypsinization, and washed twice with 10 ml of K buffer containing 25 mM HEPES, pH 7.4/115 mM potassium acetate/3.0 mM $MgCl_2$/0.9 mM $CaCl_2$ at 4°C. Permeabilization was then achieved by a two-step procedure. The cells were incubated for 10 min at 0°C with 1 ml of pre-chilled solution containing 500 units of SLO activated by dithiothreitol. After this toxin-binding step, excess toxin was removed by brief centrifugation. Cells were resuspended in 1 ml of K buffer pre-warmed to 37°C and incubated further 5 min at 37°C to cause permeabilization. Permeabilized cells were washed twice with 10 ml of ice-cold K buffer, resuspended in 2 ml transport medium (25 mM HEPES, pH 7.4/115 mM potassium acetate/2.5 mM $MgCl_2$/2.0 mM $CaCl_2$/12 mM EGTA), and incubated on ice for 15–20 min. The cells were spun down at 800× g for 5 min, resuspended in 1.0 ml transport medium, and stored on ice until further use.

Hypo-osmotic permeabilization

CHO or CHO·C2GnT cells were permeabilized by hypo-osmotic shock combined with vigorous scraping as described previously [12]. CHO cells in 10 cm tissue culture dish were washed 3 times with an ice-cold swelling buffer containing 10 mM HEPES, pH 7.2 and 15 mM KCl. After incubating 10 min on ice, the swelling buffer was removed and replaced with 3 ml of breaking buffer containing 50 mM HEPES, pH 7.2 and 90 mM KCl. Cells were immediately scraped with a rubber policeman, pelleted at 800× g for 5 min and washed once with 5 ml breaking buffer. The washed cells were resuspended in 1.0 ml transport medium and stored on ice until assayed.

Incubation of permeabilized cells with UDP-[^{3}H]Gal

Permeabilized cells resuspended in transport medium were incubated with 1 μM UDP-[^{3}H]Gal (1 μCi; 60 Ci/mmol) in a final volume of 100 μl containing 10 mM $MnCl_2$, 0.1 mM 5′-adenylylimidodiphosphate, 1 U of hexokinase, 10 mM glucose, and 0.25 mM GAP. Incubations were done at room temperature for 1 h and, unless otherwise noted. Following incubations, they were chased for an additional 30 min after adding 100 μl of a cocktail containing 100 μM each of unlabeled UDP-Gal, UDP-GlcNAc, and CMP-Sia in 10 mM $MnCl_2$, 1 U of hexokinase, 10 mM glucose in transport medium to give a final concentration of 50 μM each. The incubations were immediately centrifuged and the pellet was processed to extract glycoside products. The ethanol extracted membranes.

Extraction and purification of glycoside products from permeabilized cell incubations

A modification of the method of Etchison et al. [5] was used to extract and purify glycoside products from the permeabilized incubations. The pellet was extracted with 0.5 ml of 70% ethanol and brief sonication. The samples were centrifuged at maximum speed in a micro centrifuge for 3 min. The extraction was repeated on the pellet, and the supernatant extracts were pooled and dried *in vacuo* on a SpeedVac concentrator (Savant). The ethanol-insoluble pellets were used to determine label incorporated into protein by precipitation with 5% trichloroacetic acid. Alternatively, the material was solubilized and digested with PNGaseF to determine the amount of labeled N-linked oligosaccharides. The soluble glycoside products were purified from the dried supernatant extracts by reverse phase chromatography on C18 mini-column (0.2-ml bed volume) which had been pre-washed with 5 volumes each of absolute methanol and water, and equilibrated with 0.1 M ammonium formate, pH 6.0. The SEs were redissolved in 200 μl of 0.1 M ammonium formate and passed through the C18 mini-column. The columns were washed three times with 300 μl of 0.1 M ammonium formate followed by wash with 300 μl distilled water. The bound glycoside products were eluted with three 200 μl portions of 50% methanol.

Analysis of glycoside products

Structural analysis of glycoside products was carried out with glycosidase digestions and amine adsorption HPLC as described below.

Glycosidase digestions were done in 10–50 μl final volumes at room temperature for 16 to 20 h using the supplied buffers. All digestions were terminated by heating at 100°C for 2 min, cooling, and centrifugation. The amounts of glycosidases used per digestion, either alone or in combination, were as follows: AUS, 1 mU; NDV sialidase, 2 mU; *S. pneumoniae* β-galactosidase, 1 mU; bovine testis β-galactosidase, 5 mU; jack bean β-hexosaminidase, 50 mU; *S. pneumoniae* β-hexosaminidase, 1 mU. Glycosidase digestions were monitored as described previously [3].

Analysis of neutral and anionic glycoside products by amine adsorption analysis HPLC was carried out on a 4.5 × 250-mm Microsorb-MV (NH_2) column using an acetonitrile and ammonium formate, pH 6.0 gradient. The formate/acetonitrile gradient was started at 10 mM/80% and it was maintained for 5 min at 0.5 ml/min. The concentrations were changed to 10 mM/40% between 5 and 45 min, followed by 150 mM/40% between 45 and 75 min; then, to 250 mM/0% between 75 and 90 min. These conditions separate various neutral and anionic glycoside products containing one or two sialic acids. The same column with a gradient of 65–35% acetonitrile over 60 min was used to separate neutral glycoside products according to their size. Radioactivity was monitored using an IN/US βRAM detector.

To establish the oligosaccharide structures of the various GAP products approximately 10^6 cpm of material was fractionated using the amino column described above. Each peak was collected separately and rerun once again on the same column to assure purity. Each peak was then sequentially digested with each of the various glycosidases and then rerun on the same

column. Shifts in size or charge of the GAP product showed whether they were sensitive to the digestion. Glycosides differing by one monosaccharide were easily separated using this column.

Incorporation of [^{3}H]Gal at different temperatures

Temperature-dependent SLO-permeabilized CHO·C2GnT cell reactions were carried out with the following modifications to the standard incubation conditions without chase: 1 μM UDP-[^{3}H]GlcNAc or UDP-[^{3}H]Gal was used as the sugar donor and the reaction mixtures were incubated at different temperatures, 22°C or 37°C. The labeled glycoside products were purified and quantified as described above.

Results

Glycoside products made by intact cells

CHO cell lines were incubated with GalNAc-α-phenyl (GAP) and [^{3}H]Gal. The glycoside primed a series of labeled O-linked oligosaccharides that are secreted into the medium. The structure of each radiolabled component was determined by sequential or combined exoglycosidse digestion followed by reanalysis on HPLC, as described in the Methods (Table 1). The biosynthetic pathways for core-1 and core-2 oligosaccharides are shown in Figure 1. Normal CHO cells have only the core-1 pathway. When these cells are incubated with GAP, they synthesize and secrete neutral, mono- and disialylated chains with up to 4 monosaccharides. CHO·C2GnT expresses core-2 GlcNAc transferase, which promotes the synthesis of branched chains with 2-3 poly-*N*-acetyllactosamine repeats with up to 9 additional monosaccharides. Figure 2 shows the distribution of products recovered from the media. Each cell line made glycoside products that include all of the O-linked oligosaccharides found on endogenous proteins made by these lines [7,13].

Table 1. GalNAcαPh-based oligosaccharides synthesized by CHO and CHO·C2GnT cells

Code	*Structure*
1	Galβ1,3GalNAcαPh
2	Galβ1,3(GlcNAcβ1,6)GalNAcαPh
3	Galβ1,3(Galβ1,4GlcNAcβ1,6)GalNAcαPh
4	Galβ1,3((Galβ1,4GlcNAcβ1,3)$_{1\text{-}2}$Galβ1,4GlcNAcβ1,6)-GalNAcαPh
5	Siaα2,3Galβ1,3GalNAcαPh
6	Siaα2,3Galβ1,3(Galβ1,4GlcNAcβ1,6)GalNAcαPh
7	Siaα2,3Galβ1,3((Galβ1,4GlcNAcβ1,3)$_{1\text{-}2}$Galβ1,4GlcNAcβ1,6)GalNAcαPh
8	Siaα2,3Galβ1,3(Siaα2,3Galβ1,4GlcNAcβ1,6)-GalNAcαPh
9	Siaα2,3Galβ1,3(Siaα2,3(Galβ1,4GlcNAcβ1,3)$_{1\text{-}2}$Galβ1,4GlcNAcβ1,6)GalNAcαPh
10	Siaα2,3Galβ1,3(Siaα2,6)GalNAcαPh

The structure of each species was determined by single or multiple exo-glycosidase digestions and the products analyzed by HPLC as described in "Materials and Methods."

Glycoside products made by enriched CHO Golgi preparations

Previously, we showed that purified, intact, and correctly oriented rat liver Golgi preparations could transport and concentrate low micromolar concentrations of radiolabeled donor sugar nucleotides into the Golgi lumen, where they labeled endogenous acceptors [3,8,14]. Freely diffusable glycosides such as GAP passively enter the Golgi and are also labeled using the transported, concentrated sugar nucleotides in the Golgi lumen and resident glycosyltransferases. The majority of labeled glycosides remain within the Golgi lumen, and in some cases, their glycosylation appears to deplete the supply of transported sugar nucleotides and selectively compete out the glycosylation of resident endogenous acceptors [3].

To determine if enriched Golgi from CHO and CHO·C2GnT could synthesize glycoside products similar to those secreted from living cells, Golgi were purified using standard vigorous homogenization and centrifugation (Figure 3). The purified preparations were incubated with 1 μM UDP-[^{3}H]Gal, non-labeled UDP-GlcNAc, CMP-Sia and GAP. The sugar nucleotides are transported into the Golgi lumen increasing their concentration sufficiently for glycosylation to occur. If GAP diffuses into a Golgi compartment, it will be labeled and elongated only if the appropriate sugar nucleotide transporters and glycosyltransferases are also located in the same lumen. Further elongation of [^{3}H]Gal-labeled product requires that additional glycosyltransferases and their respective sugar nucleotide transporters be located within the same physically continuous lumen.

Following incubation, the labeled glycosides within and outside of the Golgi were measured following centrifugation of the membranes. Essentially all of the labeled GAP products (>95%) remained within the Golgi and were sedimented by centrifugation. The amount of product formed increased with increasing GAP concentration (Figure 3A). We needed to be certain that GAP labeling occurred within the Golgi and required sugar nucleotide transport. Previous studies with rat liver Golgi preparations had shown that addition of 0.1% Triton X-100 prevents the transporter-mediated concentration of the sugar nucleotide donors within the Golgi lumen. Any transported donor simply leaks out once again. Adding this detergent to the CHO cell Golgi preparations eliminated incorporation of ^{3}H into the glycosides (data not shown), indicating that Golgi integrity is required.

Intervesicular transport is very unlikely to occur under these conditions, since the Golgi preparations are essentially free of cytosol as determined by the absence of 98% of the lactic dehydrogenase activity (data not shown). Furthermore, the amount of incorporation and structure of the products is the same when the reactions were done with vesicular transport inhibitors

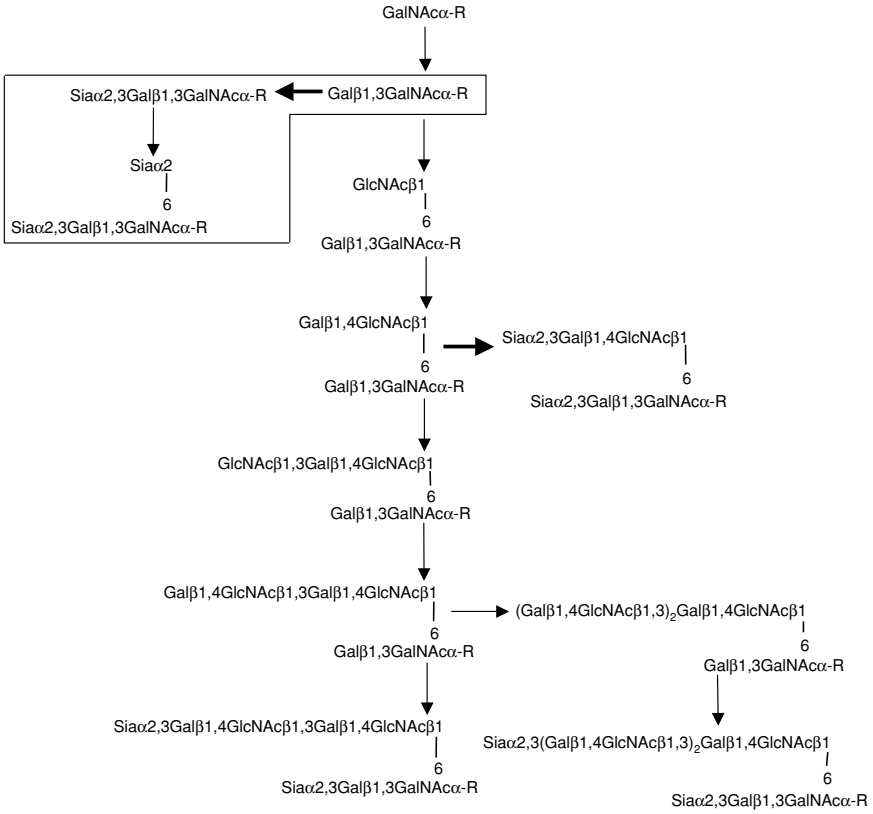

Figure 1. Biosynthetic pathway for O-glycans in wild-type CHO and CHO cells transfected with Core2GnT. R indicates the aglycone. Bold arrows indicate major products formed. Boxed area at left denotes core-1 based structures. All others require the presence of Core 2 GlcNAc transferase.

N-ethylmaleimide or ilimaquinone (data not shown). Compared to highly purified (125-fold) rat liver Golgi vesicles, the 9-fold purified CHO cell Golgi preparations have low specific activity (Table 2). Moreover, the 10–16% recovery of Golgi-associated β-galactosyltransferase activity in this multi-step purification protocol is about half of that obtained from rat liver Golgi preparations.

HPLC analysis of the *in vitro* Golgi glycoside products is shown in Figure 3B and C. CHO primarily synthesizes the trisaccharide Sia2,3Galβ1,3GalNAc-α-phenyl which is the most common product found on native O-linked chains [7] and on GAP products made by intact CHO cells. These results suggest that the galactosyl and sialyl transferases, and their donor transporters are localized within the same lumenal compartment. Golgi from CHO·C2GnT cells also make these core 1 structures, as well as a small portion of a core-2 based chains, containing Galβ1,4GlcNAc (product 6, Table 1). No extended poly-*N*-acetyllactosamine chains or disialylated products were made. Using longer incubation times or several-fold higher concentrations of sugar nucleotides do not change this profile. Taken together, these results show that core 1 Galβ1,3 transferase and Sia2,3 transferase and their sugar nucleotide transporters are functionally co-localized within the same compartment or lumen in these Golgi preparations. At least a portion of core-2 GlcNAc transferase and UDP-GlcNAc transporter must also be co-localized within the same compartment. However, the proportion of core 2 based products made in these Golgi preparations is considerably less than that seen in native O-linked chains [7,13] or in GAP products made by intact CHO·C2GnT cells.

In contrast to rat liver Golgi preparations [3], CHO cell Golgi preparations label endogenous acceptors very poorly. The amount was consistently 20–50 fold less than that found in GAP-products (Table 2). The yield of endogenous products was insufficient for analysis, and we could not assess whether glycosides selectively inhibit glycosylation of endogenous acceptors. This shortcoming together with the low yield of Golgi protein following the lengthy purification protocol, necessitated the search for a simpler and mechanically less insulting method to prepare Golgi with higher glycosylation efficiency. We reasoned that a gentler disruption system is more likely to preserve the fragile Golgi architecture seen in electronphotomicrographs [15,16]. A more rapid and efficient system would allow us to assess the compartmental distribution of the glycoslytranferases and sugar nucleotide transporters under a variety of incubation conditions.

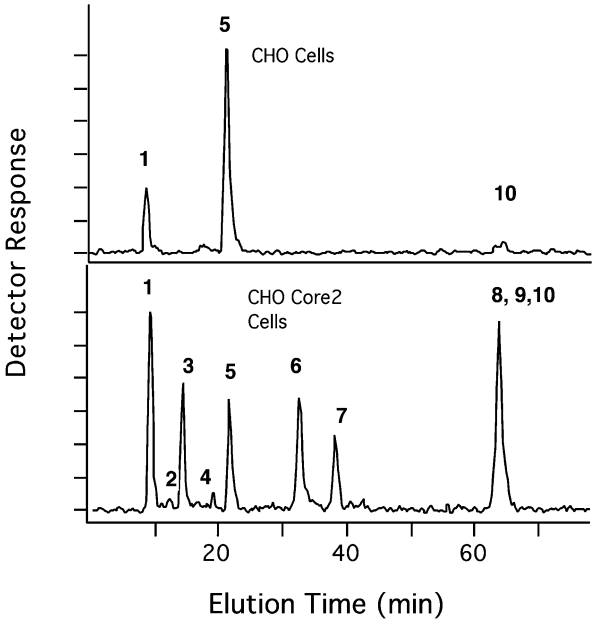

Figure 2. Amine adsorption HPLC analysis of [^{3}H]Gal-labeled GAP products synthesized and secreted by CHO-leu and CHO-leu·C2GnT cells. Cells were labeled with 250 μM GAP and the labeled glycoside products secreted into the medium were prepared and analyzed by HPLC as described in "Materials and Methods." The identity of each product was based on exoglycosidase digestions and elution position from the column. Numbers correspond to the structures in Table 1.

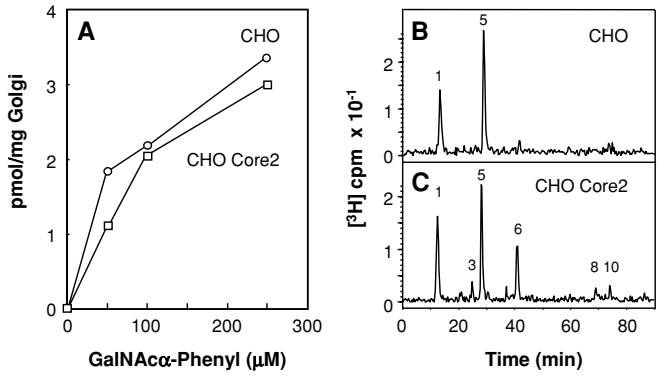

Figure 3. Analysis of glycoside products made in the presence of different concentrations of GAP. Intact Golgi from CHO(-○-) or CHO·C2GnT (-□-) were incubated with 1 μM UDP-[^{3}H]Gal in the presence of 0–250 μM GAP (Panel A) using incubation conditions described in "Materials and Methods". The glycoside products were extracted and quantified as described [5]. Extracted glycoside products from the Golgi pellet of CHO-leu (Panel B) or CHO-leu·C2GnT (Panel C) were analyzed by amine adsorption HPLC. The numbers correspond to the structures in Table 1.

Glycoside products made by permeabilized cells

We selected two related approaches to study the organization of functional glycosyltransferases and sugar nucleotide transporters in the Golgi. These methods were selected because they are rapid, avoid harsh mechanical disruption, and use only low-speed centrifugation. Controlled streptolysin-O permeabilization of cells creates pores in the plasma membrane of up to 30-nm that allow soluble molecules of <150 kDa to exit the cell [17,18]. Hypotonic lysis produces cells with large and irregular holes in the plasma membrane [12] without disrupting the internal structures. Both methods have been extensively used to study cytosolic factor and ATP requirements for Golgi vesicular transport *in vitro*. CHO and CHO·C2GnT cells were >95% permeabilized using streptolysin-O or hypo-osmotic shock as measured by loss of LDH activity or trypan-blue staining.

SLO-permeabilized CHO·C2GnT cells were incubated with 250 μM GAP for various times at 22°C or 37°C and labeled with either UDP-[^{3}H]Gal or UDP-[^{3}H]GlcNAc at 1 μM (Figure 4). The amount of glycoside product was proportional to the concentration of GAP added. Essentially all of the GAP products (95–100%) remained within the Golgi and were sedimented by low speed centrifugation of the permeabilized cells. Addition of 0.1% Triton X-100 during incubation reduced the incorporation

Table 2. Incorporation of [^{3}H]Gal into Golgi prepared by different methods

		Specific activity (pmol/mg protein)			
Sample	*Purification GalT*	*Glycosides*	*RGE*[4]	*Endogenous acceptors*	*RGE*[4]
Rat liver[1]	125	70	0.6	25	0.2
CHO[1]	9	10–25	1.1–2.8	0.5	0.05
CHO (SLO)[2]	3.4	3–9	0.9–2.6	4–8	1.2–2.4
CHO (Hypo)[3]	2.3	4–7	1.7–3.0	1–2	0.4–0.9

Purified Golgi or permeabilized cells were incubated with 250 μM GAP and 1 μM UDP-[^{3}H]Gal as described under "Materials and Methods". The products of each reaction were purified and quantified by calculating the picomoles of [^{3}H]Gal incorporated into the glycoside or endogenous acceptors per mg protein. Fold purification is based on increase in β1,4 galactosyl transferase (GalT) specific activity.
[1] Rat liver and CHO cells prepared by homogenization [5].
[2] Streptolysin-O permeabilized CHO cells.
[3] Semi-permeabilized CHO cells.
[4] Relative glycosylation efficiency (RGE) is calculated by dividing the specific activity by fold purification.

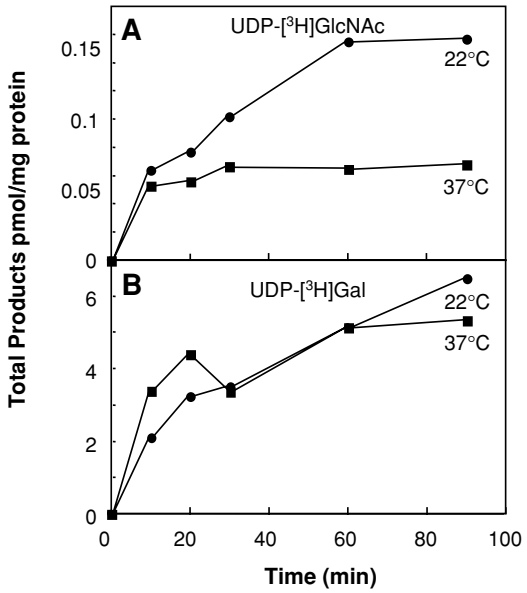

Figure 4. Time course of incorporation of [^{3}H]GlcNAc or [^{3}H]Gal into glycoside products at different temperatures. SLO permeabilized CHO·C2GnT cells were incubated with 250 μM GalNAcαPhenyl and 1 μM UDP-[^{3}H]GlcNAc (Panel A) or UDP-[^{3}H]Gal (Panel B) at different temperatures 22°C (-●-) or 37°C (-■-). The labeled glycoside products were purified and quantified as described in Figure 3.

into GAP-products by at least 20-fold, showing that sugar nucleotide transport and concentration within the Golgi lumen was essential for their synthesis. Vesicular transport is not involved in the synthesis of the glycoside products, since depletion of ATP with glucose and hexokinase (glucose + ATP → glucose-6-P) or addition of GTPγS, which blocks vesicular transport do not change the amount or identity of the products made (data not shown).

In contrast to the purified Golgi preparations, both SLO and semi-permeabilized cells incorporate substantial amounts of label into endogenous acceptors. In the case of SLO-permeabilized cells, the amount of endogenous product (4–8 pmoles/mg protein) was approximately equal to the amount of GAP products formed during the incubation. In semi-permeabilized cells, the endogenous products were approximately one-third the amount of glycoside products. The somewhat more vigorous methods used to prepare permeabilized cells by osmotic shock might account for the lower glycosylation of endogenous acceptors in comparison to controlled SLO-permeabilization. In each case the endogenous labeled acceptor proteins were resistant to proteinase digestion, indicating that they reside within the Golgi lumen and are protected from proteolysis (not shown).

About 5% of the UDP-[^{3}H]Gal added at the beginning of the incubation was transferred to glycoside and endogenous products. The remaining label was recovered as UDP-[^{3}H]Gal, showing it was not degraded. The consumption of transported donors in the synthesis of GAP products within the Golgi lumen did not inhibit or compete out the glycosylation of endogenous acceptors, which were mostly found to be *N*-linked oligosaccharides based on PNGaseF digestion (data not shown). The labeling efficiency of both GAP and endogenous products, combined with the simplicity, gentleness, and reproducibility of the permeabilization make these methods preferable over the multi-step, low yield results using harsh homogenization.

These results show that SLO and hypo-osmotic shock produce substantial labeling of both endogenous acceptors and GAP. Labeling requires sugar nucleotide transport and GAP products are completely retained within membranous compartments that sediment on centrifugation. Labeling does not require conditions needed for vesicular transport. In these experiments, the glycosylation machinery is in a static condition and glycosylation depends only on GAP, sugar nucleotide donors transported prior to or during the incubation, and the donor and acceptor affinities of the respective transferases.

Structural analysis of GAP-oligosaccharides made by permeabilized cells

Although GAP itself freely diffuses through the cell and Golgi membranes, larger labeled GAP products remain membrane bound and are sedimented by centrifugation. To insure that the multiple glycosylation steps did not involve diffusion of GAP products between different vesicles, UDP-[^{3}H]Gal labeling was done in the presence of 0.1 U/ml of *Arthrobacter ureafaciens* sialidase (AUS) which has a broad substrate specificity. Sialylated molecules not protected within a vesicle would be rapidly hydrolyzed to non-sialylated derivatives. As shown in Figure 5A, there was very little loss (<10%) of sialylated

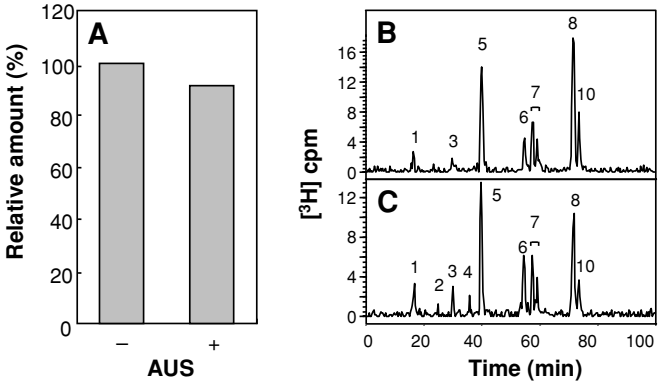

Figure 5. Verification of Golgi intactness using *Arthrobacter ureafaciens* sialidase (AUS). Osmotically permeabilized CHO·C2GnT cells were incubated (see "Materials and Methods") with or without 10 mU unit of AUS in 100 μl. Total [^{3}H]Gal labeled glycoside products made in the presence of AUS were extracted, quantified and compared to those without AUS, which is defined as 100% (Panel A). Panel B shows the amine absorption HPLC profile of the products without AUS; Panel C, the same analysis of those with AUS. The numbers correspond to the structures in Table 1.

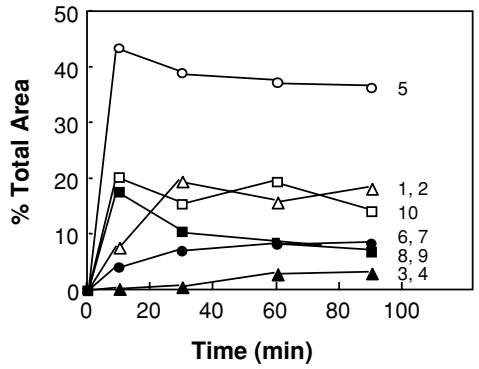

Figure 6. Time course of GAP products in SLO-permeabilized CHO·C2GnT cells. The [^{3}H]Gal labeled GAP products made from 10 to 90 min incubation from Figure 4 (22°C of Panel B) were analyzed by amine absorption HPLC. Each peak area is expressed as a percentage of total peak area on the chromatogram. The numbers indicate the corresponding structures as described in Table 1.

molecules. Moreover, the HPLC profiles of glycoside products synthesized in the absence (Figure 5B) or presence (Figure 5C) of AUS were nearly identical.

The relative proportion of different GAP products vs. time is shown in Figure 6. These results indicate that the biosynthetic reactions were rapid under these incubation conditions. Fully sialylated products rapidly appear and then decrease somewhat during prolonged incubations. Since these changes occur within protein-impermeable vesicles, it suggests that the Golgi may contain an intra-lumenal sialidase.

The efficient glycosylation of cells prepared by both SLO and osmotic shock made it possible to test different incubation conditions. For both CHO and CHO·C2GnT, incubation with only UDP-[^{3}H]Gal, but no other sugar nucleotides, produced some sialylated molecules (Figure 7A and E). This means that sialylation relied on previously transported CMP-Sia. In addition, CHO·C2GnT cells made GlcNAc-extended core 2 molecules, showing that previously transported UDP-GlcNAc was also available within the Golgi lumen. If the UDP-[^{3}H]Gal labeling was subsequently chased for 30 min with unlabeled 5 μM UDP-Gal, CMP-Sia and UDP-GlcNAc, the proportion of more highly sialylated core 1 and extended core 2 molecules was increased, showing that previously transported sugar nucleotides were limiting (Figure 7B and F). If all three sugar nucleotides were added at the beginning of the incubation period, the glycosides were almost exclusively disialylated core-1 and core-2 structures (Figure 7C and G). An additional chase period of 30 min made little difference in the profile (Figure 7D and H).

Digestion of products with AUS showed that the disialylated species contained 2-3 poly-*N*-acetyllactosamine repeats in cells expressing core-2 GlcNAc transferase (Figure 8). A similar proportion of repeats was also found during shorter incubation times with UDP-[^{3}H]Gal. These chains were as large

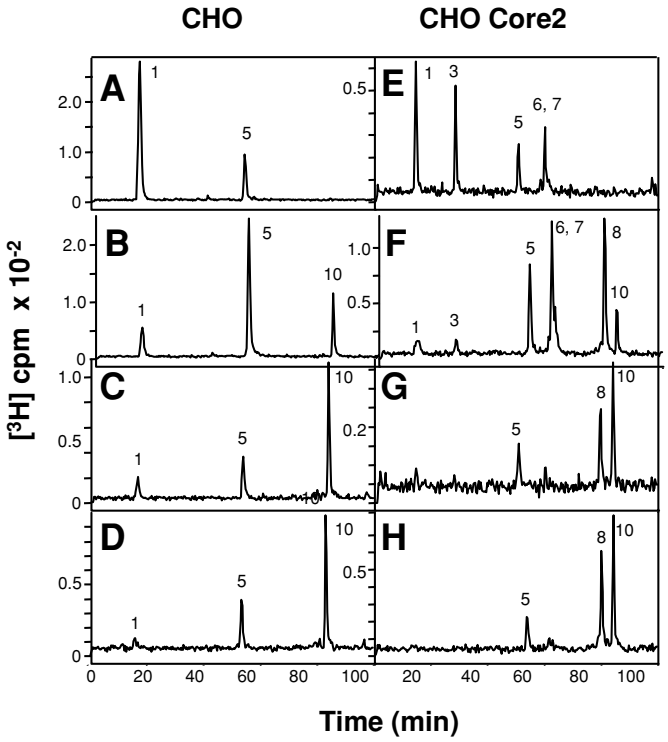

Figure 7. Amine adsorption HPLC analysis of [^{3}H]Gal labeled glycoside products made by permeabilized CHO cells. SLO permeabilized CHO or CHO·C2GnT cells were incubated with 250 μM GAP and 1 μM UDP-[^{3}H]Gal (Panel A and E), then chased with unlabeled sugar nucleotides as detailed in Materials and Methods (Panels B and F). The same cells were incubated with 1 μM UDP-[^{3}H]Gal/UDP-GlcNAc/CMP-Sia (Panels C and G) then chased as above (Panels D and H). The numbers indicate the corresponding structures in Table 1.

as those normally found on native glycoproteins or those made by live CHO·Core2 cells incubated with GAP. This is significant because it indicates that 9 separate glycosyltransferase reactions can sequentially modify the same GAP molecule within a single continuous lumen that is functionally defined as the Golgi.

CHO appeared to make almost exclusively disialylated (70%) and mono-sialylated oligosaccharides (25%) with a minor amount of neutral molecules (5%) as shown in Figure 7D. These were predicted to be Sia2,3Galβ1,3(Sia2,6)GalNAcα-phenyl and Sia2,3Galβ1,3GalNAcα-phenyl, respectively. Digestion with AUS collapsed all to the disaccharide Galβ1, 3GalNAcα-phenyl, showing that all charge was due to sialic acids. To confirm the structure, we also digested the samples with Newcastle disease virus (NDV) sialidase, which preferentially cleaves Sia2,3Gal linkages. The specificity of this enzyme preparation was confirmed by digesting a known standard with the enzyme. NDV sialidase digested the disialylated species to a molecule containing a single negative charge and it digested the mono-sialylated molecule to a neutral molecule.

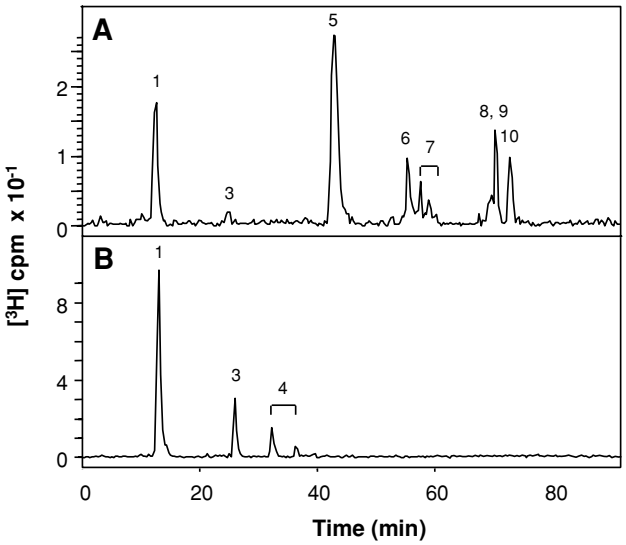

Figure 8. Amine adsorption HPLC analysis of [^{3}H]Gal labeled glycoside products obtained after AUS digestion. A portion of the glycoside products made by osmotically permeabilized CHO·C2GnT was run either before (Panel A) or after digestion (Panel B) with AUS. The numbers correspond to the structures in Table 1.

Permeabilized cells can use a GlcNAc-β-benzyl primer

The above results suggest that permeabilized cells have all the enzymes and sugar nucleotide transporters needed to produce GAP products as complex as those made by the respective intact cells. We asked whether other glycosides could also be extended using these *in vitro* conditions. Intact CHO cells incubated with GlcNAc-β-benzyl, synthesize mostly Sia2,3Galβ1,4GlcNAc-β-benzyl, and $\sim$15% of the sialylated products have one or two poly-*N*-acetyllactosamine repeats [19]. Osmotically lysed CHO cells incubated with GlcNAc-β-benzyl also made mostly (57%) Sia2,3Galβ1,4GlcNAc-β-benzyl while 6% of all chains had one additional poly-*N*-acetyllactosamine unit. Thus, GlcNAc-β-benzyl products also resemble those made by intact cells. Nearly the same proportion of sialylated and poly-*N*-acetyllactosamine-containing chains are made regardless of the addition of CMP-Sia during the incubation, suggesting that most of the sialylation draws on previously transported CMP-Sia.

Discussion

In the current studies, we wanted to know if Golgi derived from CHO cells containing core-2 GlcNAc transferase could make GAP products that resembled those made by live cells incubated with GAP. The "freeze frame" method used by Hayes et al. [8] and Hayes and Varki [14] and adapted by Etchison et al. [5] for glycoside acceptors allowed us to manipulate the incubation conditions. Cells containing Core-2 GlcNAc transferase make oligosaccharides on glycoproteins that are mostly core-2 based, some have poly-*N*-acetyllactosamine repeats [20]. When a purified Golgi fraction was incubated with the appropriate sugar nucleotide donor and GAP, the synthesis of glycoside products showed that nucleotide transporters and some glycosyltransferases resided in the same functional compartment. However, very little core-2-dependent products were made, despite finding that cells labeled with [^{3}H]Gal secreted GAP products with up to 9 added monosaccharides. Also, in contrast to studies from rat liver Golgi preparations, these Golgi preparations also showed very little incorporation into endogenous proteins. This may be because they did not co-reside in the same vesicle with the transferases or because the conditions used for preparation or incubation were not optimal.

Vigorous homogenization disrupts the Golgi, reducing the average number of saccules seen in electron photomicrographs [21]. We considered the possibility that the low yield and limited synthesis of Core2-dependent products might be due to physical disruption of fragile Golgi connections between enzymes early in the pathway and Core2 enzyme. To preserve the potentially labile features, we permeabilized the cells with SLO or osmotic shock. These methods deplete cytosolic proteins and small molecules such as ATP. Since vesicular transport does not occur without cytosolic factors and an energy source, glycosylation within a lumenal compartment is controlled by adding donor and acceptor substrates, exactly as done for conventionally purified Golgi. Glycosylation efficiencies using these methods for primers were as good as the more vigorous methods, but were much simpler to use (Table 2) and labeling of endogenous acceptors was 10–20 fold higher than that obtained using homogenization. The results showed that permeabilized cells could be used to study glycosylation of artificial acceptors or endogenous proteins.

The cisternal maturation model of protein glycosylation and secretion has enjoyed a resurgence in popularity [22–24]. Time-lapse video imaging of GFP-tagged Golgi proteins [25], and biochemical analyses of the critical components [26,27] visualizes the Golgi as a highly dynamic assembly of vacuoles, tubules and vesicles, rather than as a set of static compartments and a series of shuttling cargo-loaded vesicles [2]. Confining glycosyltransferases exclusively to the *cis*-, *medial*-, *trans*-Golgi or *trans*-Golgi network compartments as separate domains was probably not accurate, but many studies show selective, but not exclusive, localization of transferases to these named domains. Serial section or scanning electron microscopy of Golgi within individual cells sometimes showed a single continuous and interconnected network of intermittent and perhaps transient tubular connections [15,16]. Our results provide biochemical evidence that glycosylation reactions normally thought to occur in different Golgi compartments can actually occur within a single continuous lumenal compartment. Up to 9 sequential biosynthetic steps of the O-linked oligosaccharide biosynthetic pathway can occur within a single lumenal compartment that must contain all the glycosyltransferases and sugar nucleotide transporters required for the addition of these monosaccharides.

Many endogenous acceptors are expected to be membrane bound and might not diffuse through the lumenal space as rapidly as the glycoside acceptors. This is, of course, speculation.

Another interpretation of our results is that distinct Golgi compartments have different average steady state populations of transferases, but that each lumenal compartment contains a sufficient amount of all glycosyltransferase and sugar nucleotide transporters to yield fully mature GAP products. This is possible, but we do not know how much enzyme is required to give the amount of products we see. Regardless of which view is taken, the extent of GAP glycosylation defines a functional compartment based on accessibility of a freely diffusable molecule within a lumenal space, irrespective of whether this consists of a single large compartment or a series of smaller, vesicles and interconnecting tubules. Mechanical disruption as seen in this study, natural physiological processes such as mitosis [29,30], or drugs that induce Golgi fragmentation [9,27,31] will create discontinuities. Depending on how the discontinuity was created, it may alter or limit glycosylation by segregating various glycosyltransferases from each other or from their sugar nucleotide transporters.

These simple, quantitative, and sensitive methods could track the functional continuity of the Golgi resident transporters and transferases. This can be easily adapted to various settings simply by selecting the appropriate glycosides, transfected CHO cell lines, and labeled sugar nucleotides. It should be possible to simultaneously analyze the functional co-localization of many glycosyltransferases and sugar nucleotide transporters in the Golgi at any time, independent of the morphological appearance of the Golgi. Functional glycosylation analysis can complement studies of Golgi organization using epitope- or GFP-tagged [25,26,32], proteins, and other biochemical markers [33,34].

Acknowledgments

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