Brefeldin A promotes the appearance of oligosaccharyl phosphates derived from Glc₃Man₉GlcNAc₂-PP-dolichol within the endomembrane system of HepG2 cells

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Abstract We reported an oligosaccharide diphosphodolichol (DLO) diphosphatase (DLODP) that generates dolichyl-phosphate and oligosaccharyl phosphates (OSPs) from DLO in vitro. This enzyme could underlie cytoplasmic OSP generation and promote dolichyl-phosphate recycling from truncated endoplasmic reticulum (ER)-generated DLO intermediates. However, during subcellular fractionation, DLODP distribution is closer to that of a Golgi apparatus (GA) marker than those of ER markers. Here, we examined the effect of brefeldin A (BFA), which fuses the GA with the ER on OSP metabolism. In order to increase the steady state level of truncated DLO while allowing formation of mature DLO (Glc₃Man₉GlcNAc₂-PP-dolichol), dolichyl-P-mannose Man₉GlcNAc₂-PP-dolichol mannosyltransferase was partially downregulated in HepG2 cells. We show that BFA provokes GA endomannosidase trimming of Glc₃Man₉GlcNAc₂-PP-dolichol to yield a Man₉GlcNAc₂-PP-dolichol structure that does not give rise to cytoplasmic Man₉GlcNAc₂-P. BFA also strikingly increased OSP derived from mature DLO within the endomembrane system without affecting levels of Man₉GlcNAc₂-PP-dolichol or cytoplasmic Man₉GlcNAc₂-P. The BFA-provoked increase in endomembrane-situated OSP is sensitive to nocodazole, and BFA causes partial redistribution of DLODP activity from GA- to ER-containing regions of density gradients. These findings are consistent with BFA-provoked microtubule-dependent GA-to-ER transport of a previously reported DLODP that acts to generate a novel endomembrane-situated OSP population. — Massarweh, A. M. Bosco, S. Iatmanen-Harbi, C. Tessier, L. Amana, P. Busca, I. Chantret, C. Gravier-Pelletier, and S. E. H. Moore. Brefeldin A promotes the appearance of oligosaccharyl phosphates derived from Glc₃Man₉GlcNAc₂-PP-dolichol within the endomembrane system of HepG2 cells. J. Lipid Res. 2016. 57: 1477–1491.

The biosynthesis of oligosaccharyl diphosphodolichol (DLO) that is required for protein N-glycosylation is initiated on the cytoplasmic face of the endoplasmic reticulum (ER) by the glycosylation of the lipid anchor, dolichyl phosphate (DolP), to form Man₉GlcNAc₂-PP-dolichol (1–3). This intermediate is subsequently flipped onto the luminal face of the ER membrane where it is further glycosylated to yield the mature DLO, Glc₃Man₉GlcNAc₂-PP-dolichol (1). After oligosaccharyltransferase-mediated transfer of Glc₃Man₉GlcNAc₂ onto protein, the remaining luminal oriented dolichyl phosphate is recycled to yield cytoplasmically facing DolP (4). These reactions constitute the dolichol cycle and in certain types of congenital

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Abbreviations: ALG12, the gene encoding dolichyl-P-mannose Man₉GlcNAc₂-PP-dolichol mannosyltransferase; AP, aminopyridine; BFA, brefeldin A; CDG, congenital disorder of glycosylation; CNX, calnexin; Con A, concanavalin A; CST, castanospermine; DLO, oligosaccharyl diphosphodolichol; DLODP, oligosaccharyl diphosphodolichol diphosphatase; DolP, dolichyl phosphate; endoH, endo-B-N-acetylglucosaminidase H; ER, endoplasmic reticulum; ERGIC, endoplasmic reticulum/Golgi apparatus intermediate compartment; fOS, free oligosaccharide; GA, Golgi apparatus; GCA, galactosidase A; GlcNAc, N-acetylgalactosamine; IMQ, ilimaquinone; KIF, kifunensine; MAN1A1, Golgi apparatus mannosidase I; MAN1B1, endoplasmic reticulum mannosidase I; MAN2A1, Golgi apparatus mannosidase II; MANEA1, Golgi apparatus endomannosidase; MBC, membrane bound compartment; mOS, neutral free oligosaccharide; NZ, nocodazole; OSP, oligosaccharyl phosphate; QAE, quaternary aminoethyl; RPNI, ribophorin I; SLO, streptolysin O; SW, swainsonine; UGT, UDP-galactose glycoprotein galactosyltransferase.

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disorders of glycosylation (CDGs) the cycle is disrupted and protein hypoglycosylation ensues (5). DLO levels are controlled in several ways, including hydrolytic reactions that lead to the destruction of DLO and generation of free oligosaccharides (OIs) (6–13). When the dolichol cycle is interrupted and truncated, cytoplasmically oriented, DLO intermediates (\(\text{Man}_{2}\text{GlcNAc}_{2}\text{PP-dolichol}\)) accumulate, cytoplasmic oligosaccharyl phosphates (OSPs) with corresponding structures (\(\text{Man}_{2}\text{GlcNAc}_{2}\text{P}\)) are observed (7, 14). Using semi-intact cells derived from a patient diagnosed with dolichyl-P-mannose\(\text{Man}_{2}\text{GlcNAc}_{2}\text{PP-dolichol}\) mannosyltransferase deficiency (ALG12-CDG), in which the DLO intermediate, \(\text{Man}_{2}\text{GlcNAc}_{2}\text{PP-dolichol}\), accumulates, \(\text{Man}_{2}\text{GlcNAc}_{2}\text{P}\) is liberated from a pool of \(\text{Man}_{2}\text{GlcNAc}_{2}\text{PP-dolichol}\) (15). Surprisingly, the released \(\text{Man}_{2}\text{GlcNAc}_{2}\text{P}\) is also predominantly recovered in the cytosolic compartment, despite the fact that \(\text{Man}_{2}\text{GlcNAc}_{2}\text{PP-dolichol}\) is generated in the lumen of the ER (15). To further complicate understanding of DLO regulation, \(\text{Glc}_{2}\text{Man}_{4}\text{GlcNAc}_{2}\text{P}\) structures have been detected in yeast membranes (16) and at low levels in mammalian cells (17). However, in patients with ALG6-CDG or ALG8-CDG where \(\text{Glc}_{1}\text{Man}_{2}\text{GlcNAc}_{2}\text{PP-dolichol}\) structures accumulate, increases in \(\text{Glc}_{1}\text{Man}_{2}\text{GlcNAc}_{2}\text{P}\), similar to the increases of OSP seen in cells from CDG patients where more truncated (\(\text{Man}_{2}\text{GlcNAc}_{2}\text{PP-dolichol}\)) DLO accumulates, are not observed (14, 15). The ensemble of these observations suggests that OSP generation is a complex phenomenon and indicates multiple sources of OSP.

Presently, the machinery underlying OSP production has not been characterized at the molecular level, and how an OSP generation mechanism might distinguish between normal biosynthetic DLO intermediates, on the one hand, and, on the other, those that are surplus to requirements or possess aberrant structures is not understood. Recently, a Co \textsuperscript{2+}-activated liver microsomal DLO diphosphatase (DLODP) activity that splits both mature and truncated DLO to yield OSP and DolP has been reported (18). This reaction might enable DolP recycling from unproductive or potentially dangerous DLO intermediates that occur in CDG. However, the potential physiological role of this enzyme in DLO regulation remains uncertain because, during density gradient centrifugation, this activity distributes similarly to a marker of the Golgi apparatus (GA), and differently to those of ER-situated DolP-dependent DLO biosynthetic enzymes (18). The complexity of this data prompted us to evaluate the subcellular compartmentalization and membrane topology of OSP production in human hepatocellular HepG2 carcinoma cells treated with drugs that perturb the endomembrane system. Here we show that brefeldin A (BFA), which causes fusion of the GA with the ER, provokes both the appearance of a truncated DLO intermediate that does not give rise to a cytoplasmic OSP, and complex GA-modified OSP within the endomembrane system. By contrast, in ALG12-deficient HepG2 cells, BFA did not affect the levels of either \(\text{Man}_{2}\text{GlcNAc}_{2}\text{PP-dolichol}\) or cytoplasmic \(\text{Man}_{2}\text{GlcNAc}_{2}\text{P}\) that accumulated in this cell model of ALG12-CDG. These data demonstrate at least two independent OSP pools and suggest that there is more than one mechanism for OSP generation.

**MATERIALS AND METHODS**

**Reagents**

D-[2-\textsuperscript{3}H(N)]mannose (24.7 G Ci/mmol) and EN\textsuperscript{2}HANCE™ spray were from PerkinElmer Life Sciences (Zaventem, Belgium). Uridine 5’-diphosphate [1-\textsuperscript{3}H]galactose (15–30 G Ci/mmol) was from BIOTREND Chemikalien GmbH (Koln, Germany). Silica-coated TLC plates were from Merck (Darmstadt, Germany). Dowex resins, fucose, 2-acetamido-2-deoxy-1,3,4,6-tetra-\(\text{O}\)-acyetyl-D-glucopyranose, endo-\(\beta\)-N-acetylglucosaminidase H (endoH) from *Streptomyces plicatus*, Jack bean \(\alpha\)-mannosidase, Jack bean \(\beta\)-hexosaminidase, protease inhibitor cocktail, protease, nocardazole (NZ), BFA, golgicide A (GGA), Carestream\textsuperscript{®} Kodak\textsuperscript{®} X-Omat LS film, OptiPrep\textsuperscript{™} and alkaline phosphatase were purchased from Sigma-Aldrich SARL (St. Quentin Fallavier, France). Castanospermine (CST), kifunensine (KIF), and swainsonine (SW) were from Toronto Research Chemicals Inc. (Toronto, Canada). The antibodies used were: anti-Golgi apparatus mannosidase II (MAN2A1; Thermo Fisher product number PA5-18851), anti-ribophorin I (RPN1; Sigma-Aldrich product number SAB2102853), anti-calnexin (CNX; BD Biosciences product number 610524), anti-formiminodolyltransferase cyclodeaminase was from Sigma-Aldrich (product number G2404). Stealth siRNA duplexes, nontargeting stealth RNAi negative medium GC control duplex, reduced serum medium (Opti-MEM \textsuperscript{®}), Lipofectamine\textsuperscript{®} RNAiMAX, NuPAGE\textsuperscript{®} LDS sample buffer, Absolute\textsuperscript{™} Blue QPCR SYBR\textsuperscript{®} Green Mix, BCA\textsuperscript{™} protein assay kit, and the Verso cDNA synthesis kit were purchased from Thermo Scientific (Courtaboeuf, France). RNeasy\textsuperscript{®} mini kit and RNase-free DNase I were obtained from Qiagen (Courtaboeuf, France). The Luminol ECL detection system was from Millipore (Billerica, MA). Streptolysin O (SLO) was purchased from Sucharit Bahkdi, Johannes-Gutenberg-Universität, Mainz, Germany. Illimaquinone (IMQ) was from Santa Cruz Biotechnology, Dallas, TX.

**Cell culture and RNAi transfection**

HepG2 cells, CHO cells, and the Thy\textsuperscript{-1} DPMI-deficient mouse lymphoma cell line (19) were from ATCC (Rockville, MD), and were cultivated in RPMI 1640 Glutamax™ medium containing 10% fetal calf serum and 1% penicillin/streptomycin. All cells were cultivated at 37°C under an atmosphere containing 5% CO\textsubscript{2}. Control nontargeting (medium GC) and ALG12-targeted (sense 1, 5’ AGACCUGGGACGAGUAGCGCAUCU 3’; sense 2, 5’ CCGGGUUUACGUGCUUUUGGU 5’; sense 3, 5’ UAGCCGCUUUGCCGCAUACUGGUUGA 3’) stealth siRNA duplexes were transiently transfected into HepG2 cells using Lipofectamine RNAiMax as previously described (20).

**RNA extraction and quantitative RT-PCR**

RNA extraction and reverse transcription were performed using the RNeasy mini kit and the Verso cDNA synthesis kit, respectively, according to the manufacturer’s instructions. For quantitative (Q) PCR, specific ALG12 (sense, GCCAGTGGT-GATGCCGAC; reverse, CGAGTCCAAGACCTCTCTAA) and S14 housekeeping gene primers (sense, TCAGTCGAAAGAATACCATTTG; reverse, CGAGTTTCTGATTACACGGAC) were used.
Metabolic radiolabeling of cells

Cells were incubated in glucose-free RPMI 1640 medium containing 0.5 mM glucose, 2.0 mM fucose, and 2% dialyzed fetal calf serum and different reagents (detailed in each figure legend) for 1 h. Then 100 μCi/ml [2-3H (N)]mannose was added and the incubations continued for a further 2 h. For generation of Glc5(α1→2)Man,GlcNAc2,PP-dolichol, 8 × 10−7 M Thy− cells were harvested and then rinsed with glucose-free RPMI 1640 medium containing 0.5 mM glucose, 2.0 mM fucose, and 2% dialyzed fetal calf serum and then incubated in 1 ml of the same medium containing 20–100 μCi [2-3H (N)]mannose for 30 min.

Permeabilization of HepG2 cells

Specific permeabilization of plasma membranes with SLO was performed as described previously (21): cells were incubated at 4°C with 0.5 ml precooled permeabilization buffer [5 mM HEPES (pH 7.0) containing 250 mM mannitol and 2 mM CaCl2] containing 2 μg/ml SLO. After 1 h, the SLO-containing medium was removed from the cells and combined with a subsequent 1 ml permeabilization buffer wash.

Recovery of DLO, OSP, neutral fOS, and glycoproteins from cells and medium

These methods are based on previously described procedures (6, 22). Washed cells were suspended in 4 ml of methanol/100 mM Tris-HCl (pH 7.4) containing 4 mM MgCl2, 2:1. Four milliliters of CHCl3 were added and the mixture shaken. After centrifugation, three phases were obtained: a water/methanol upper phase, a lower CHCl3 phase, and an interphase containing pre- precipitated protein and certain DLO. DLO was recovered from the interphase material. The two organic phases were pooled and collected and the protein precipitate was washed once with 3 ml of methanol. The two supernatants were combined and centrifuged at 750 g for 5 min, the supernatant was collected and the pellet was again homogenized and centrifuged, as above, to give a pellet (P1). The two supernatants were combined and centrifuged at 6,000 g for 10 min and 100,000 g for 45 min to give P2 and P3, respectively, and a final supernatant (S). OptiPrep solutions were diluted into 10 mM HEPES/NaOH (pH 7.4) containing 250 mM sucrose. P3 was made 20% with respect to OptiPrep and layered onto a 30% OptiPrep cushion before overlaying with 15% OptiPrep. The tubes were centrifuged at 350,000 g for 90 min using a Beckman VTi 65.2 rotor.

Analytical procedures

Charged oligosaccharides were desalted on Biogel P2 columns prior to fractionation on quaternary aminoethyl (QAE)-Sephadex columns (25), as previously described (15). Neutral oligosaccharides were separated on silica-coated plastic sheets (0.2 mm thickness) developed in n-propanol/acetic acid/water (3:3:2) for 16–24 h (26). Radioactive components were detected on X-OMAT AR film by fluorography after spraying the dried TLC plates with Enhance and were quantitated by scintillation counting after their elution with water from the silica. Where indicated, fluorographs were scanned and images quantified using the BIO-1D software (Vilber Lourmat, Germany). Where appropriate, data were analyzed and plotted using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA; http://www.graphpad.com). Oligosaccharides were derivatized with 2-aminopyridine (AP), separated by normal-phase and reversed-phase HPLC, and detected by on-line fluorometry, as previously described (27). OSPs were analyzed by concanavalin A (Con A)-Sepaharose chromatography exactly as previously described (28). The characterization of oligosaccharide structures using alkaline phosphatase, jack bean α-mannosidase, jack bean β-hexosaminidase, and endo-β-N-acetylglucosaminidase was carried out according to the protocols supplied by Sigma-Aldrich. Protein was detected using the BCA reagent (29). UDP-galactosyl glycoprotein galactosyltransferase (UGT) was assayed as previously described (30). For the DLODP assay: Glc5(α1→2)Man,GlcNAc2-PP-dolichol (16 × 10−7 cm) was dried down into 1.3 ml centrifugation tubes before being resuspended in 5 μl 1% NP-40. Then further components were added to give a final reaction mixture of 100 mM MES (pH 5.5), 1 mM CoCl2, 0.1% NP-40, 100 μM SW, 100 μM KIF, 1 mM CFT, and 4.2–8.4 μg protein in a volume of 50 μl. Incubations were carried out at 37°C for 20 min and processed as described in (18).

Western-blot analysis

Samples prepared from density gradient fractions were dialyzed to the same protein concentration, heated with NuPAGE LDS sample buffer under reducing conditions according to the manufacturer’s instructions, and subjected to SDS-PAGE; the primary antibodies were detected using horseradish peroxidase-coupled secondary antibodies.

Density gradient fractionation of HepG2 cell homogenates

HepG2 cell monolayers (80% confluent) were homogenized and fractionated according to a protocol devised for rat liver (23) with some modifications. Cells were washed twice with ice-cold PBS and scraped into ice-cold homogenization buffer [10 mM HEPES/NaOH (pH 7.4) containing 250 mM sucrose and mammalian protease inhibitors]. To estimate the cell volumes, the scraped cells were centrifuged at 200 g at 4°C for 5 min, and then the total volume was adjusted to give 4 vol of homogenization buffer for each volume of cells. Cells were homogenized by 20 strokes in a loose fitting Dounce homogenizer. After centrifugation at 750 g for 5 min, the supernatant was collected and the pellet was again homogenized and centrifuged, as above, to give a pellet (P1). The two supernatants were combined and centrifuged at 6,000 g for 10 min and 100,000 g for 45 min to give P2 and P3, respectively, and a final supernatant (S). OptiPrep solutions were diluted into 10 mM HEPES/NaOH (pH 7.4) containing 250 mM sucrose. P3 was made 20% with respect to OptiPrep and layered onto a 30% OptiPrep cushion before overlaying with 15% OptiPrep. The tubes were centrifuged at 350,000 g for 90 min using a Beckman VTi 65.2 rotor.

RESULTS

Whereas some studies support the idea that OSPs are hydrolyzed from truncated DLO intermediates on the cytoplasmic face of the ER, others suggest that OSPs can be
derived from mature DLO within the ER. Recently we reported on a Co 

2+-activated DLODP activity that liberates OSps from both truncated and mature DLO, but that does not codistribute with Dol-P-dependent enzymes of the dolichol cycle during density gradient centrifugation of microsomal membranes (18). In fact, the bulk of this DLODP activity distributed similarly to GA-situated galactosyltransferase (UGT) (18). These results prompted us to examine the subcellular sites of OSP production in living cells using drugs that perturb the endomembrane system (Fig. 1A). The modes of action of several commonly used drugs to dissect out ER and GA function are shown in Fig. 1A. Both NZ and IMQ cause fragmentation and dispersal of the GA throughout the cytoplasm without affecting GA-dependent protein secretion (31, 32). Whereas it is known that NZ primarily disrupts microtubules required for GA stabilization (33), the molecular target for IMQ has not yet been determined, but its effects on GA structure seem to require phospholipase D (34). BFA promotes fusion of elements of the GA with the ER to form a composite structure (35, 36). Within this structure, glycoprotein processing normally associated with the cis, medial, and, to a lesser extent, trans GA can continue (37, 38), but glycoprotein exit does not occur (39). This ER/GA composite structure (Fig. 1A) has been termed the BFA compartment (38). Recently GCA has been shown to have similar effects on cultured cells to those of BFA (40). Thapsigargin inhibits the sarco/ER Ca2+-ATPase that is involved in maintenance of the ER Ca2+ pool (41) and is known to inhibit ER-associated glycoprotein processing and also glycoprotein secretion (42). In order to examine the effect of perturbing the endomembrane system on DLO and OSP generation, HepG2 cells were preincubated with either carrier or the different drugs for 1 h prior to metabolic radiolabeling with [2-3H]mannose for 2 h in the continued presence of drugs. TLC examination of oligosaccharides derived from DLO and OSP generated under these conditions is shown in Fig. 1B. In untreated cells, DLO occurs mainly as Glc3Man9GlcNAc2-PP-dolichol. Small amounts of OSP whose glycans structures comigrate with Man5,6GlcNAc2 and Glc3Man9GlcNAc2 are detected. The smaller OSP structures are observed in different cell types grown under normal conditions and in increased amounts in cells with blocks in the dolichol cycle where truncated Man7,8GlcNAc2,PP-dolichol intermediates accumulate (10, 14, 15). Much less is known about the origins of OSps that are derived from larger DLO (16, 17). As shown in Fig. 1B, IMQ and NZ did not have a striking effect on the thin-layer chromatographic profiles of the oligosaccharides derived from either the DLO or OSP. By contrast, both BFA and GCA provoke the appearance of a truncated DLO, whose oligosaccharide moeity (Fig. 1B, a) comigrates with Man9GlcNAc2, and a family of OSP structures (Fig. 1B, b–f), whose glucan moieties do not comigrate with standard polymannose-type structures. TG caused an increase in radioactivity associated with DLO along with small changes in the profiles of oligosaccharides derived from both DLO and OSP, but did not provoke the striking changes seen with either BFA or GCA. The effects of the latter agents

Fig. 1. BFA fuses elements of the GA with the ER and perturbs DLO metabolism. A: In the ER glycoprotein N-glycans (NG) are deglucosylated by ER glucosidase I (GLS1) and glucosidase II (GLS2), and MAN1B1. After vesicular transport to the cis GA, some deglucosylated N-glycans are deglucosylated by MAN1A1. Subsequent to transport to the medial GA, N-glycans are modified by N-acetylgalactosaminyltransferase I (GNT1), then MAN2A1, and finally by other N-acetylgalactosaminyltransferases (such as GNT2). N-glycoletylproteins are then transported to the trans GA where N-glycans are galactosylated by UGT. Finally, after vesicular transport to the trans Golgi network (TGN), N-glycans are sialylated by sialyltransferases (ST) before being packaged into vesicles for transport to their final destinations. BFA and GCA cause the cis, medial, and trans GA to fuse with the ER to form the BFA or GCA compartment (red dashed line). NZ and IMQ cause the GA to fragment and disperse throughout the cytoplasm. Thapsigargin (TG) blocks Ca2+ transport into the ER and perturbs many aspects of ER function as well as protein secretion. The ER glucosidase I and II inhibitor, CST, the MAN1B1 and MAN1A1 inhibitor, KIF, and the MAN2A1 inhibitor SW are used to understand N-glycan processing reactions in the endomembrane system. B: HepG2 cells were treated with the carrier, DMSO, 20 μM IMQ, 10 μg/ml NZ, 4 μg/ml BFA, 10 μM GCA, or 100 nM TG as indicated, before metabolic radiolabeling with [2H]mannose for 2 h in the continued presence of the drugs. DLO and OSP were extracted and examined by TLC as described in the Materials and Methods. The migration positions of Glc3Man9GlcNAc2 (G), Man9GlcNAc2 (M), Man9GlcNAc2 (M), and Man9GlcNAc2 (M) are indicated to the left of the chromatogram.
were chosen for further study because, although a truncated DLO became apparent, similar to the situation seen in cells from certain patients with CDG, the corresponding OSP was not observed, and instead, a population of novel OSP structures (Fig. 1B, b–f) was detected.

**Golgi endomannosidase processing of DLO in BFA-treated cells**

In order to further investigate the glycan structure of the BFA-induced DLO (Fig. 1B, a), HepG2 cells were treated with BFA for 3 h prior to harvesting and extracting DLO with organic solvents. Oligosaccharides were released from DLO, derivatized with the fluorophore, 2-AP, and resolved by HPLC as shown in Fig. 2A. As expected from data shown in Fig. 1B, the HPLC profiles reveal that, in the presence of BFA, the predominant DLO possesses an oligosaccharide moiety (labeled a) with an elution time corresponding to that of Man₉GlcNAc₂-AP. Material eluting under peak a in Fig. 2A could correspond to Man₉GlcNAc₂-AP or a glucosylated structure containing the same number of hexoses, such as, for example, Glc₆Man₉GlcNAc₂-AP or Glc₅Man₉GlcNAc₂-AP. To resolve this issue, peak a (Fig. 2A) material was recovered and digested with jack bean α-mannosidase in order to determine whether any of its α-linked mannose residues were shielded by glucose residues. Greater than 95% of the starting material was converted into Man₁₄GlcNAc₂-AP, demonstrating that the predominant species eluting under peak a (Fig. 2A) is not glucosylated and therefore contains eight residues of mannose (A. Massarweh et al., unpublished observations). As shown by the scheme in Fig. 2B, accumulation of Man₉GlcNAc₂-PP-dolichol could be the consequence of a block in the addition of the ninth mannose residue during DLO biosynthesis, resulting in the accumulation of the normal (d₁,d₂)Man₉GlcNAc₂-PP-dolichol biosynthetic intermediate. Alternatively, Man₉GlcNAc₂-PP-dolichol could be demannosylated by either ER- or GA-situated mannosidases to yield (d₁,d₃)Man₉GlcNAc₂-PP-dolichol. Finally, GA endomannosidase (MANEA1; see Fig. 1A), which can only act on glucosylated glycans (43), could deglucosylate Glc₆Man₉GlcNAc₂-PP-dolichol to yield (d₂,d₃)Man₉GlcNAc₂-PP-dolichol. To distinguish between these possibilities, DLO were examined by TLC as described in the Materials and Methods; the migration positions of Glc₅₆Man₉GlcNAc₂ (G₅₆M₉), Man₉GlcNAc₂ (M₉), and Man₉GlcNAc₂ (M₉) are indicated to the left of the chromatogram.

**Fig. 2.** Characterization of DLO structures generated in the presence of BFA. A: HepG2 cells were treated with 2.5 μg/ml BFA or the appropriate amount of carrier for 1 h prior to harvesting and extraction with organic solvents to yield DLO-containing CHCl₃ and CHCl₃/methanol/water (10/10/3) phases. After acid hydrolysis of DLO, released oligosaccharides were derivatized with 2-AP and resolved by normal-phase HPLC. The elution positions of Glc₆Man₉GlcNAc₂-AP (G₃M₉), Glc₆Man₉GlcNAc₂-AP (G₃M₉), Glc₅Man₉GlcNAc₂-AP (G₅M₉), Man₉GlcNAc₂-AP (M₉), Man₅GlcNAc₂-AP (M₅), and Man₆GlcNAc₂-AP (M₆) are indicated. Peak a was collected and characterized as described below. B: A DLO intermediate containing eight residues of mannose could arise through several mechanisms. First, the normal DLO biosynthetic route (solid blue arrows) yields (d₁,d₂)Man₉GlcNAc₂-PP-dolichol. Second, two catabolic processes could potentially generate different structures (dashed blue arrows): 1) MANEA1 could cleave the tetrasaccharide, Glc₆Man, from Glc₆Man₉GlcNAc₂-PP-dolichol to yield (d₂,d₃)Man₉GlcNAc₂-PP-dolichol; 2) MANIB1 could remove a single mannose residue from Man₉GlcNAc₂-PP-dolichol to yield (d₁,d₃)Man₉GlcNAc₂-PP-dolichol. C: HepG2 cells were pretreated with 2.5 μg/ml BFA or the appropriate amount of carrier and the indicated glycosidase inhibitors (100 μM SW, 100 μM SW + 100 μM KIF, or 100 μM SW + 100 μM KIF + 2 mM CST) for 1 h before metabolic radiolabeling with [2-³H]mannose and incubating for a further 2 h. DLO were extracted and oligosaccharides derived from Oligosaccharide diphasphodolichol hydrolysis
experiments were conducted in the presence of the mannosidase and glucosidase inhibitors described in Fig. 1A. Neither control nor BFA-induced DLO profiles were affected by the presence of the MAN2A1 (Fig. 1A) inhibitor SW (Fig. 2C). By contrast, whereas addition of the ER mannosidase I (MAN1B1; Fig. 1A) and MAN1A1 (Fig. 1A) inhibitor, KIF, did not affect the DLO profile seen in the absence of BFA, it blocked the appearance of the BFA-induced species containing seven hexose units without affecting the quantity of component a (Fig. 2C). Finally, the addition of the glucosidase I and II inhibitor, CST (Fig. 1A), as well as both KIF and SW, to the cells caused an increase in the proportion of triglucosylated DLO in both control and BFA-treated cells, but did not completely block the appearance of the BFA-provoked DLO bearing eight hexose residues (Fig. 2C). These data suggest that MANEA1, which is insensitive to CST, KIF, and SW (43) and is known to hydrolyze glucosylated DLO in vitro (44), might underlie the appearance of Man$_6$GlcNAc$_2$-PP-dolichol in BFA-treated cells. Next, the Man$_6$GlcNAc$_2$AP structure (Fig. 2A, HPLC peak a) was digested with S. pilcatus endo-β-N-acetylglucosaminidase H (endoH) to yield Man$_6$GlcNAc, which was then rederivatized with AP and subjected to reversed-phase HPLC with a standard mixture of Man$_n$GlcNAc$_m$-AP isomers, as shown in Fig. 2D. The chromatographs demonstrate that the (d2,d3)Man$_6$GlcNAc$_2$ isomer that is the hallmark of endomannosidase action is the major oligosaccharide structure associated with Man$_6$GlcNAc$_2$-PP-dolichol derived from BFA-treated cells. Finally, Man$_6$GlcNAc$_2$-PP-dolichol is not observed in KIF/CST/BFA-treated Chinese hamster ovary (CHO) cells (Fig. 2E) that are known to be sensitive to BFA (45), but express an inactive MANEA1 (46). The ensemble of these data demonstrates that glucosylated DLO species are trimmed by MANEA1 in BFA-treated HepG2 cells.

**BFA induces the appearance of a novel family of OSPs in HepG2 cells**

In order to understand why the appearance of Man$_8$GlcNAc$_2$-PP-dolichol is not accompanied by the appearance of Man$_9$GlcNAc$_2$-P in BFA-treated cells, OSPs isolated from the glucosidase-treated cells described in Fig. 2C were examined by TLC. In the absence of BFA, as shown in Fig. 3A (left panel), irrespective of the presence of glucosidase inhibitors, Glc$_3$Man$_n$GlcNAc$_2$ is detected along with the smaller components migrating as standard Man$_8$GlcNAc$_2$ that were described in Fig. 1B. When SW and KIF are added to the cell cultures, the OSP profiles are modified to a small extent. Upon addition of CST to the inhibitor mix, a population of larger structures migrating between Glc$_3$Man$_n$GlcNAc$_2$ and Man$_9$GlcNAc$_2$ is noted in addition to the smaller structures. In the absence of glucosidase inhibitors, BFA provokes the appearance of a complex mixture of OSPs (Fig. 1B and right panel of Fig. 3A, components b–f). Upon addition of SW to the BFA-treated cells, the OSP profile changes and two major species (right panel of Fig. 3A, components g and h) are observed. When BFA-treated cells are radiolabeled in the presence of both KIF and SW, only OSPs bearing oligosaccharide structures migrating as standard Man$_9$GlcNAc$_2$ are observed, despite the occurrence of glucosylated DLO in this condition (compare right panel of Fig. 3A with Fig. 2C). In the presence of CST, KIF, and SW, the OSP glycan structures are similar to those of DLO observed under the same conditions (compare right panel of Fig. 3A with Fig. 2C), comprising predominantly Glc$_3$Man$_6$GlcNAc$_2$ and Man$_9$GlcNAc$_2$ species. The changes in distributions of the novel OSP species that are not observed with the different glucosidase inhibitors suggest that BFA-provoked OSPs originate from DLO in the lumen of the BFA compartment and then undergo processing by SW- and KIF-sensitive enzymes that are normally present in the GA. The unexpected appearance of this novel population of OSPs in BFA-treated cells prompted us to confirm that these structures do, in fact, possess a phosphate moiety linked at the CI position of their reducing N-acetylglucosamine (GlcNAc) residues. To do this, cells were treated with the three glycosidase inhibitors and BFA, metabolically radiolabeled, and, after solvent extraction, total MeOH/H$_2$O upper phases were dried down and desalted on a Biogel P2 column. Ion-exchange fractionation of the desalted material on QAE-Sephadex (Fig. 3B, left panel) demonstrates that although the bulk of radioactivity is associated with previously described nOS and the tetrasaccharide (Glc$_3$Man) produced by endomannosidase (Fig. 2A, MANEA1), a peak of radioactivity is eluted with 75 mM NaCl. TLC examination of this purified charged material after further salting revealed a mixture of slow migrating components comprising structures that give rise to predominantly Glc$_3$Man$_n$GlcNAc$_2$ and Man$_9$GlcNAc$_2$, and Glc$_5$Man$_n$GlcNAc$_2$ and Man$_6$GlcNAc$_2$ after alkaline phosphatase and endoH treatments, respectively (Fig. 3B, middle panel). These data confirm that the BFA-provoked structures contain a phosphate residue linked to the reducing GlcNAc moiety of the oligosaccharide (Fig. 3B, right panel).

**Identification of β-hexosaminidase-sensitive complex-type OSPs in BFA-treated cells**

Next the glycan structure of the OSPs recovered from BFA-treated cells was examined using Con A-Sepharose chromatography. Data shown in Fig. 3C demonstrate that GA-modified complex- and hybrid-type structures constitute 80–90% of total OSP when cells are metabolically radiolabeled in the absence of glucosidase inhibitors. Upon addition of SW, the distribution of OSP changes, with hybrid-type structures becoming predominant (Fig. 3C). As expected, addition of KIF resulted in the predominance of polymannose-type OSPs. It was noted that the quantity of material associated with the 10 mM α-methyl glucose eluates from the Con A-Sepharose columns does not change strikingly when cells are treated with the glucosidase inhibitors. Because it is known that certain truncated polymannose-type oligosaccharides, such as those occurring in truncated DLO intermediates (Man$_7$GlcNAc$_2$-PP-dolichol), elute with complex bi-antennary oligosaccharides under these conditions (47, 48), it is likely that the small Man$_7$GlcNAc$_2$-P species detected in cells radiolabeled in
Inhibitors - SW KIF CST
\[ M_1 - M_2 - M_3 - \]
\[ G_M_0 - \]

BFA

Panel)

\[ M_1 - M_2 - M_3 - \]
\[ G_M_0 - \]

control (right panel) or the appropriate amount of carrier (left panel) and the indicated glycosidase inhibitors for 1 h before addition of [2-\(^{3}H\)]mannose and incubating for a further 2 h. Incubations were stopped and OSPs were recovered from the cells as described in the Materials and Methods. OSPs were dephosphorylated by heating in 20 mM HCl for 45 min and the resulting neutral oligosaccharides were examined by TLC. The migration positions of Glc3Man9GlcNAc2 (G2M0), Man5GlcNAc2(M3), Man9GlcNAc2(M7), Man5GlcNAc2(M9), and Man9GlcNAc2(M12) are indicated to the left of the chromatograms. B: Cells were radiolabeled in the presence of BFA and the glycosidase inhibitors, SW, KIF and CST, and after solvent extraction of the cells, the methanol/water phase was dried down and desalted prior to being analyzed by QAE-Sephadex ion-exchange chromatography (left panel). Radioactive material eluted from the column with 75 mM NaCl was desalted and analyzed by TLC (right panel, lane 1). The same material was treated with either alkaline phosphatase (ALP) or endoH and, after desalting, was analyzed by TLC (right panel). C: OSPs were recovered from cells treated with BFA and the indicated glycosidase inhibitors and, after elution from Dowex 1X2 columns with 3 M formic acid, were desalted on Biogel P2 columns. The desalted OSPs were submitted to Con A-Sepharose chromatography as described in the Materials and Methods. Whereas complex tri- and tetra-antennary glycans do not bind to the column, complex bi-antennary-, hybrid-, and polymannose-type glycans are eluted with 10 mM \(\alpha\)-methylglucoside, 10 mM \(\alpha\)-methylmannoside, and 500 mM \(\alpha\)-methylmannoside, respectively. The radioactivity recovered in each fraction was measured by scintillation counting and, in order to determine the relative molar amount of each type of structure, the values were divided by 3 for fractions containing complex-type glycans, by 5 for fractions containing hybrid-type glycans, and by 8.5 for fractions containing polymannose-type glycans. These molar equivalents are expressed as percentage of the total molar equivalents recovered from each condition. D: E: Total desalted OSPs (as described for panel B) recovered from cells treated with BFA alone (D) or BFA + SW (E) were treated with alkaline phosphatase (ALP) and neutral oligosaccharides, recovered after combined Dowex 1X2 and Dowex 50X2 chromatography, were digested with either jack bean \(\alpha\)-mannosidase (\(\alpha\)-Man) or jack bean \(\beta\)-hexosaminidase (\(\beta\)-Hex). The migration positions of Glc3Man9GlcNAc2(G2M0), Man5GlcNAc2(M3), Man9GlcNAc2(M7), Man5GlcNAc2(M9), Man9GlcNAc2(M12), and Man9GlcNAc2(M14) are indicated to the left of the chromatograms.

**Quantitation of OSP in control and BFA-treated cells**

In cells that have not been treated with BFA (open bars in Fig. 4A), the DLO pool is small (about 2% of total radioactivity incorporated into cells) and turns over rapidly, and under the radiolabeling conditions employed, is probably close to steady state. During this radiolabeling period, the DLO pool gives rise to radioactive N-glycosylproteins (60–70% of all radioactive components recovered from the cells and medium), nfOS (20–40% of all radioactive components recovered from the cells and medium), and the absence of BFA (Fig. 1B and left panel of Fig. 3A) also occur in BFA-treated cells and that these components contribute significantly to the radioactivity associated with the 10 mM \(\alpha\)-methyl glucose eluates. Next, in order to confirm the presence of complex and hybrid type OSPs in BFA and BFA- and SW-treated cells, respectively, OSP obtained under these two conditions were treated with alkaline phosphatase and then subjected to either jack bean \(\alpha\)-mannosidase or \(\beta\)-hexosaminidase, as shown in Fig. 3D, E. OSPs generated by cells in the absence of SW largely resisted the mannosidase and were more susceptible to hexosaminidase, yielding predominantly a digest product behaving as Man5GlcNAc2. These data are consistent with the presence of OSP containing the trimannosyl core substituted with one or more residues of GlcNAc (Fig. 3D). OSPs recovered from the SW-treated cells are moderately sensitive to \(\alpha\)-mannosidase and yield an oligosaccharide that comigrates with Man5GlcNAc2 after digestion with \(\beta\)-hexosaminidase. These data are in accordance with the presence of hybrid-type OSP in which the Man5GlcNAc2 core structure is substituted with one or more residues of GlcNAc (Fig. 3E). Previous studies with this type of structure have shown that the exposed mannose residues are somewhat resistant to jack bean \(\alpha\)-mannosidase cleavage (49). Therefore BFA provokes the appearance of GA-modified complex OSP and suggests that Man9GlcNAc2-P-dolichol could give rise to Man5GlcNAc2-P that is then further processed by GA enzymes.
OSP (0.2–0.4% of all radioactive components recovered from the cells and medium). Misfolded glycoproteins also give rise to nfOS through ER-associated degradation processes (50). The changes in nfOS levels seen with the different glycosidase inhibitors are expected because, as previously reported (21), inhibition of the cytoplasmic and lysosomal mannosidases by SW leads to the accumulation of nfOSs that are produced during glycoprotein biosynthesis. In addition, the Golgi/MAN1B1 inhibitor, KIF (51), and the ER glucosidase inhibitor, CST (52), are known to be able to perturb normal ER-associated degradation processes and are expected to alter the levels of nfOS that are generated by these mechanisms. In BFA-treated cells (hatched bars in Fig. 4A), the DLO pool sizes increase by 1.3- to 2-fold over control values, whereas there are 5- to 10-fold increases in OSP. Although the BFA-provoked increase in OSP is striking, the radioactivity associated with OSP under these conditions does not exceed 2% of the total radioactivity incorporated into components recovered from the cells and medium. The effects of BFA on the DLO and OSP pools are not accompanied by striking effects on the amounts of radioactive N-glycosylproteins and nfOS generated during the radiolabeling period. These data suggest that if OSP catabolism is occurring, it does not contribute significantly to nfOS production.

**BFA-provoked cellular OSPs do not arise due to a block in their secretion**

Why does BFA provoke the appearance of OSP from mature DLO in the lumen of the endomembrane system? There are several explanations. First, OSP could be generated constitutively in the ER and then be secreted into the extracellular medium after passage through the GA. Under these circumstances, the BFA-provoked appearance of OSP would be due to a block in OSP secretion. To test this hypothesis, OSPs were recovered from both the cells and medium of KIF + SW-treated cells radiolabeled in either the presence or absence of BFA. As shown in Fig. 4E, the amount of radioactivity recovered as OSP in the medium of cells radiolabeled in the absence of BFA cannot account for the BFA-provoked increase in cellular OSP in BFA-treated cells. Because OSPs could be dephosphorylated in the medium to yield nfOS, these neutral structures were also quantitated in the medium; but again, the difference in the quantities of nfOS found in the medium of cells radiolabeled in either the absence or presence of BFA is not sufficient to explain the BFA-provoked increase in intracellular OSPs (Fig. 4B).

**BFA-provoked OSP accumulation is partially blocked by NZ**

Next, we wanted to determine whether the BFA-promoted appearance of OSP is the result of, first, BFA-mediated inhibition of a normal degradative process that clears potential constitutively generated OSP from the lumen of the ER, or of, second, either the BFA-provoked creation of a nonphysiological OSP generation process or a BFA-promoted increase in a normally occurring phenomenon. For example, BFA-provoked OSP might be caused by mixing of a GA-situated DLODP activity with ER-situated DLO in a manner similar to that known to occur for the BFA-provoked increase in sphingomyelin biosynthesis, where BFA is thought to cause mixing of GA-situated sphingomyelin synthase with ER-situated ceramide (31). Alternatively, a block in ER export from the BFA compartment could trap a putative secretory OSP-generating enzyme in the ER, where it could hydrolyze DLO. In order to answer these questions, we sought to determine whether BFA-provoked OSP generation was due to a simple block of vesicular transport out of the ER, or whether retrograde movement of elements of the GA into the ER is required. To do this, we examined the effect of NZ on the ability of BFA to provoke the appearance of OSP. As mentioned earlier, the organization of the GA requires microtubules and, as shown in Fig. 5A, NZ disrupts GA morphology, leading to the production of functional GA-derived vesicles that distribute throughout the cell cytoplasm. Although BFA-provoked redistribution of the GA to the ER requires intact
**Fig. 5.** Effect of NZ pretreatment on BFA-provoked OSPs. A: Scheme showing the effects of NZ on microtubules and the morphology of the GA in the presence or absence of BFA. B: Cells were treated with either NZ (20 μg/ml) or DMSO for the indicated times (pretreatment time). Subsequently, the cells were treated with SW + KIF + CST and BFA (2 μg/ml) or (DMSO) in radiolabeling medium, containing NZ or DMSO where appropriate, then 1 h later, radiolabeled as described in Fig. 2C. Glycoproteins recovered from the cells and medium were quantitated by scintillation counting after pronase digestion. The quantity of radioactivity associated with medium glycoproteins is expressed as a percentage of the total radioactivity recovered from cell and medium glycoproteins. C: The radioactivity incorporated into cellular and medium N-glycans and DLO derived from NZ-treated cells of the experiment described in (B). D (left panel): OSPs recovered from cells pretreated with NZ for 0 and 4 h before radiolabeling conducted in the presence of the glycosidase inhibitors and BFA were examined by TLC. The abbreviations used are as described for Fig. 3A. D (right panel): In two independent experiments conducted in the presence of SW + KIF + CST and BFA, where cells underwent 0, 2, and 4 h NZ pretreatment, OSPs were resolved by TLC. After fluorography, radioactive components were quantitated by densitometry. In these experiments control incubations, conducted in the presence of SW + KIF + CST, in which cells were not pretreated with NZ, were radiolabeled in the presence of BFA, but not NZ (conditions described in Fig. 1B). The quantity of OSP determined in this condition is set at 100%. The mean and standard error of the mean are shown. E (left panel): OSPs recovered from cells pretreated with NZ for 0 and 4 h before radiolabeling, conducted in the absence of the glycosidase inhibitors, but in the presence of BFA, were examined by TLC. The abbreviations used are as described for Fig. 3A. E (right panel): After fluorography, radioactive components were quantitated by densitometry, and the ratio of the quantity of OSPs in NZ pretreatment conditions to that of BFA alone (set at 100%) is presented.

**Examining the effects of BFA on the subcellular localization of Co2+-activated DLODP activity**

One possible explanation for the NZ-sensitivity of BFA-provoked appearance of OSP is that the retrograde transport of a normally GA-resident DLODP activity into the ER is required. Previous studies employing density gradient fractionation of mouse liver microsomes have indicated the presence of a Co2+-activated DLODP activity that codistributes with GA-situated UGT (see Fig. 1A). Whereas UGT-containing membranes derived from homogenates of normally cultivated cells are separated from heavier membranes of the ER during density gradient centrifugation, those derived from homogenates of BFA-treated cells partially shift to denser regions of density gradients that reduce BFA-provoked fusion of the GA with the ER (33), while not affecting the ability of BFA to block transport out of the ER or pre Golgi compartments. As expected, therefore, under our experimental conditions, pretreatment of cells with NZ for up to 4 h before the radiolabeling period does not inhibit glycoprotein secretion (compare NZ hatched bars with DMSO hatched bars in Fig. 5B). In addition, NZ pretreatment has only a minor effect on the capacity of BFA to block glycoprotein secretion (compare DMSO solid bars with NZ solid bars in Fig. 5B). Data shown in Fig. 5C demonstrate that NZ pretreatment, before the addition of BFA, does not reduce the incorporation of radioactivity into either DLO or N-glycans. TLC of the BFA-provoked OSP, after 0 or 4 h NZ pretreatment, is shown when cells are radiolabeled in the presence of the glycosidase inhibitors (Fig. 5D, left panel). Quantitation of OSP recovered from two experiments is shown in Fig. 5D (right panel), and reveals that when BFA and NZ are added to the cells at the same time (0 h NZ), there is no inhibition of BFA-provoked OSP when compared with OSP quantitated in the absence of NZ (BFA alone), but after 2 and 4 h of NZ pretreatment there is a 40–50% reduction in BFA-provoked OSP. It was also noted that pretreatment of cells with NZ caused a change in the distribution of the BFA-provoked OSP. After 4 h NZ pretreatment, the ratio of Man3GlcNAc2-P to Glc3Man9GlcNAc2-P is reduced when cells are pretreated with NZ (left panel of Fig. 5D). A similar experiment was performed in the absence of glycosidase inhibitors and, as shown in Fig. 5E, preincubation of cells for both 0 and 4 h with NZ reduces the appearance of BFA-provoked complex-type OSP. Accordingly, the BFA-provoked appearance of OSP is partially inhibited by pretreating the cells with NZ for 2–4 h before addition of BFA, and suggests that microtubules are required for this process.
contain ER markers (53). Accordingly, we asked whether the Co\textsuperscript{2+} -activated DLODP activity could be redistributed to ER-containing fractions during density gradient fractionation of BFA-treated HepG2 cells. Data shown in Fig. 6A demonstrate that UGT and DLODP cofractionate in the absence of BFA, and after BFA treatment both activities partially redistribute to heavier membranes containing the ER markers, CNX and RPN1, as shown in Fig. 6B. As has been reported previously, BFA can cause a shift of ER marker proteins to lighter regions of density gradients (54). The BFA-provoked shift in density of DLODP- and UGT-containing membranes is less pronounced than that observed for either medial GA-situated MAN2A1 (Fig. 6C) or formimidoyltransferase cyclodeaminase (Fig. 6D), whose precise location within the GA is less well-characterized. Accordingly, taken along with the metabolic radiolabeling experiments described above, these subcellular fractionation studies demonstrate that a mechanism for the BFA-provoked increase of OSP involving microtubule-dependent redistribution of a GA DLODP activity to the ER cannot be excluded.

**Fig. 6.** Density gradient fractionation of control (Ctrl) and BFA-treated HepG2 cell homogenates. HepG2 cells were treated with 10 \( \mu \)g/ml BFA for 1 h prior to harvesting and preparation of microsomes, as described in the Materials and Methods. The microsomes were submitted to OptiPrep density gradient fractionation. A: UGT and DLODP activities recovered from the gradient fractions were summed and the percentages of the total recovered in each fraction are shown. B-D: The ER markers, RPN1 and CNX, and the GA markers, MAN2A1 and formimidoyltransferase cyclodeaminase (FTCD), were quantitated by densitometry after SDS-PAGE and Western blot. The results are presented as described above. E: An OptiPrep gradient was generated in the absence of microsomal protein and the UV absorbance of each fraction was measured. The density of each fraction was calculated by comparing these absorbancies to those of OptiPrep solutions of known concentration and density.

**Fig. 7.** OSP generation in a HepG2 model for ALG12-CDG. A: Cells were transduced with either control siRNA (nt) or duplexes 1–3 targeting ALG12. ALG12 mRNA was estimated by QPCR and its quantity is expressed as percentage of that occurring in the cells transfected with the nt siRNA. B: Cell growth in (A) was estimated by total cell protein. C: Every 2 days after transfection with ALG12 siRNA 2 or nt siRNA, cells were metabolically radiolabeled with [2-\( ^3 \)H]mannose for 30 min, as described in the Materials and Methods. Following solvent extraction of the cells, the DLO recovered from the CHCl\textsubscript{3} and 10/10/3 organic phases were analyzed separately. DLO and OSP were subjected to acid hydrolysis with 20 mM HCl and the released oligosaccharides were analyzed by TLC as described in the Materials and Methods. The migration positions of Glc\textsubscript{3}-0Man\textsubscript{9}GlcNA\textsubscript{2} (G3-0M9), Man\textsubscript{7}GlcNA\textsubscript{2} (M7), and Man\textsubscript{5}GlcNA\textsubscript{2} (M5) are indicated to the left of the chromatograms.

**BFA has little effect on cytoplasmic OSP production from truncated DLO**

Next, we wanted to explore the relationship between the BFA-provoked OSPs and those generated from truncated DLO species. To this end, a HepG2 cell model of ALG12-CDG was generated by downregulating ALG12 expression in order to provoke the accumulation of Man\textsubscript{γ-GlcNAc2-PP- dolichol.} The time courses of inhibition of ALG12 mRNA expression and cell growth, after transduction of cells with three ALG12-targeting and one non-targeting siRNAs, are shown in Fig. 7A and Fig. 7B, respectively. Whereas all ALG12-targeting siRNAs inhibit ALG12 mRNA expression maximally between 2 and 4 days post transfection, cell growth is maximally affected between 4 and 8 days post transfection. Further experiments were performed with ALG12-targeting siRNA (2), whose ability to downregulate ALG12 mRNA expression was found to be the least transient of the three siRNAs. Metabolic radiolabeling experiments revealed that...
the accumulation of Man\textsubscript{3}GlcNAc\textsubscript{2}-PP-dolichol in ALG12-
downregulated cells coincided with the inhibition in cell growth noted between 4 and 8 days post transfection (Fig. 7C). Whereas large OSPs were below the limit of detection in control-transfected cells, the appearance of the OSP, Man\textsubscript{3}GlcNAc\textsubscript{2}-P, coincided with the accumulation of Man\textsubscript{3}GlcNAc\textsubscript{2}-PP-dolichol in cells transfected with ALG12-
targeting siRNA. Next, glycosidase inhibitor-treated, ALG12-
downregulated cells were metabolically radiolabeled in either the presence or absence of BFA, as described in Fig. 1B. As seen in Fig. 8A, BFA provokes the appearance of Man\textsubscript{3}GlcNAc\textsubscript{2}-PP-dolichol and Man\textsubscript{3}GlcNAc\textsubscript{2}-P as well as Glc\textsubscript{3}Man\textsubscript{3}GlcNAc\textsubscript{2}-P, without having a major effect on the quantity of radioactivity associated with either Man\textsubscript{3}GlcNAc\textsubscript{2}-PP-dolichol or Man\textsubscript{3}GlcNAc\textsubscript{2}-P. We showed that the BFA-promoted appearance of OSP is sensitive to NZ (Fig. 5B). By contrast, data in Fig. 8B show that in ALG12-
downregulated cells, neither DLO nor OSP is affected by treating the cells with NZ before radiolabeling. In a similar experiment to that described in Fig. 8A, cell plasma membranes were selectively permeabilized with the bacterial pore-forming protein, SLO, at the end of the radiolabeling period, to yield an SLO perfusate (cytosol fraction: Cyt) and a residual cellular fraction comprising intracellular membrane bound compartments (MBCs). As expected from our previously reported findings using cells from an ALG12-CDG patient, Man\textsubscript{3}GlcNAc\textsubscript{2}-P is recovered mainly in the cytosolic compartment when cells are radiolabeled in the absence of BFA (left panel of Fig. 8B) and this situation is largely unaffected when cells are radiolabeled in the presence of BFA (right panel of Fig. 8B). By contrast, the BFA-provoked OSPs are predominantly recovered from the MBC. The ensemble of these results demonstrates that BFA provokes the appearance of a population of OSPs within the endomembrane system that is distinct from that generated constitutively when truncated DLO accumulates (15).

**DISCUSSION**

Here we examined DLO and OSP metabolism in cells treated with drugs that are known to perturb membrane flux along the endomembrane system. BFA and GCA, two agents known to fuse elements of the GA with the ER, were shown to have similar effects on DLO and OSP metabolism. For the first time, we show that BFA promotes MANE1 processing of DLO. The function of MANE1 is not clear, although it is thought that this enzyme deglucosylates glucosylated glycoproteins that leave the ER after escaping ER glucosidases. The endomannosidase-mediated removal of the dsI mannose residue (see Fig. 2B) means that reglucosylation of these N-glycans by ER-situated UDP-Glc glycoprotein glucosyltransferase cannot occur. Accordingly, it has been speculated that MANE1 could play a role in the glycoprotein quality control system, during which UDP-Glc glycoprotein glucosyltransferase-tagged misfolded glycoproteins are bound to either CNX or calreticulin to promote folding (55). Our results suggest that endomannosidase could potentially also regulate DLO. Immuno-gold electron microscopy has shown that although 85% of endomannosidase is found associated with the cis GA, 15% is found to be associated with the cis GA network or ER/GA intermediate compartment (ERGIC) (55). Many proteins recycle between the ER and ERGIC.
and it is not inconceivable that, in the absence of BFA, ERGIC-situated endomannosidase transits through the ER where it could potentially act on glucosylated DLO and/or glycoproteins. Alternatively, glucosylated DLO intermediates that escape the ER could be rapidly de-glucosylated in the ERGIC or cis GA as part of a catabolic pathway. Nevertheless, we cannot rule out the possibility that the endomannosidase-generated (d2,d3) Man₉GlcNAc₂-PP-dolichol intermediate could be slowly remannosylated by the ALG9 gene product that adds the d2 and d3 terminal nonreducing mannose residues (see Fig. 2B) to the growing DLO in the lumen of the ER. In the light of our results, it will be interesting to evaluate the role of endomannosidase in DLO regulation in normally cultivated cells.

We also show for the first time that BFA promotes the appearance of complex type OSPs within the endomembrane system. A high proportion of these OSPs are shown to possess terminal nonreducing GlcNAc residues consistent with their processing by enzymes that are normally found in the cis and medial GA. Such structures have also been shown to predominate on glycoproteins trapped within the BFA compartment (37, 38). These observations suggest a generalized deficiency in galactosylation by normally trans GA-situated UGT in the BFA compartment (see Fig. 1A). Using fluorescent microscopy, it has been shown, in many cell types, that BFA causes fusion of cis, medial, and trans elements of the GA with the ER to form the BFA compartment (Fig. 1A). By contrast, the trans Golgi network was shown to fuse with elements of the endosomal system. Why is glycoprotein and OSP galactosylation reduced in BFA-treated cells? There is a progressive acidification of organelles of the endomembrane system from pH 7.2 in the ER to pH 6.0 in the trans Golgi network (56), and studies show that GA-situated glycosyltransferases form biologically important homo- and heterodimers at lower pH (57, 58). Therefore enzymes normally resident in distal GA cisternae are perhaps less active in the BFA compartment, whose pH is likely to be similar to that of the ER (59).

What is the relationship between the BFA-promoted OSPs and other OSP populations?

The oligosaccharide structures found in the different OSP pools merit discussion because they throw light on the compartmentalization and membrane topology of the OSP-generating processes. Studies using cells harboring mutations in genes encoding enzymes of the dolichol cycle demonstrate that only when DLO structures containing seven or fewer residues of mannose accumulate are there concomitant increases in the corresponding OSP structures (7, 14, 15). Either OSPs are not produced from larger DLO structures in increased amounts or, if they are, they are rapidly metabolized. Previously, we proposed that the selectivity of this OSP generating process is not related to the selectivity of the OSP generating enzyme, but is due to the capacity of the accumulated DLO to be exposed to the cytoplasmic face of the ER (15). Because DLO structures containing less than five residues of mannose are synthesized on the cytoplasmic face of the ER, any block in their synthesis might be expected to give rise to cytoplasmic OSPs (7, 14). By contrast, Man₉GlcNAc₂-PP-dolichol is generated in the lumen of the ER and how this structure gives rise to cytoplasmic OSP is not clear. It is possible that, after generation within the lumen of the ER, Man₉GlcNAc₂-PP could be rapidly and efficiently transported into the cytosolic compartment. Nevertheless, in vitro experiments using semi-intact cells derived from a ALG12-CDG patient demonstrated that while Man₉GlcNAc₂-PP was recovered predominantly in the cytosol, nOS and glycosylated acceptor peptide generated in the same experiments remained predominantly within the lumen of the ER (15).

In the face of this data, it was proposed that if Man₉GlcNAc₂-PP-dolichol accumulates in the lumen of the ER, it could potentially be flipped back onto the cytoplasmic face of the ER, where it could be hydrolyzed to yield cytoplasmic Man₉GlcNAc₂-PP (15). Indeed, protein-mediated Man₉GlcNAc₂-PP-dolichol flipping across artificial membranes has been demonstrated, but was shown to be much slower than the flipping of Man₉GlcNAc₂-PP-dolichol, and about as efficient as the flipping of Man₉GlcNAc₂-PP-dolichol (60). Thus, data from these in vitro flippase assays do not support the idea that the selectivity of the DLO flippase could regulate access of luminaly generated DLO intermediates to a cytoplasmically oriented OSP-generating mechanism. Nevertheless, it is quite possible that, in vivo, the selectivity of lumen-to-cytoplasm DLO flipping is somewhat different to that observed in artificial membranes in vitro. In the present study, we show that BFA promotes the accumulation of Man₉GlcNAc₂-PP-dolichol and that, in this situation, cytoplasmic Man₉GlcNAc₂-PP is not detected. In the presence of BFA and glycosidase inhibitors, Man₉GlcNAc₂-PP is detected within the BFA compartment, but at present we do not know whether Man₉GlcNAc₂-PP is derived directly from Man₉GlcNAc₂-PP-dolichol hydrolysis or whether Glc₃Man₉GlcNAc₂-PP is cleaved from Glc₃Man₉GlcNAc₂-PP-dolichol and subsequently hydrolyzed by MANEA1 to yield Man₉GlcNAc₂-PP. The latter hypothesis would predict that the OSP-generating mechanism within the BFA compartment might be selective for mature tri-glucosylated DLO and that BFA might amplify the OSP generation from mature DLO that has been previously noted to occur at low levels in various cell types (17). However, at present, it is not possible to say whether the BFA-promoted OSP population described here has the same origin as that of the previously described OSP population that appears to be derived from mature DLO.

Whatever the origins of the larger OSP structures seen in control or BFA-treated cells, their relationship to the cytosolic structures generated from truncated DLO intermediates containing seven or fewer residues of mannose is clearer. Here, we show that despite striking increases in OSPs derived from mature DLO within the BFA compartment, the generation of Man₉GlcNAc₂-PP from Man₉GlcNAc₂-PP-dolichol is largely unaffected by BFA. Furthermore, we show that, in the presence of BFA, Man₉GlcNAc₂-PP is still recovered from the cytoplasmic compartment of cells, even though the BFA-provoked OSPs are recovered from
within the endomembrane system. Finally, we report that whereas the BFA-promoted appearance of luminal OSPs is partially blocked by NZ, the generation of Man$_2$GlcNAc$_2$-P from Man$_3$GlcNAc$_2$-PP-dolichol is not. These data point to independent processes for the generation of these two OSP pools.

**Why does BFA promote the appearance of complex type OSPs within the endomembrane system?**

There are two general explanations: BFA either blocks the catabolism or secretion of constitutively generated OSPs, or it increases OSP generation. The first explanation is that OSPs are normally generated from mature DLO within the membrane system, but in the absence of BFA they are either secreted into the extracellular medium or are rapidly further metabolized and difficult to detect. We show that in the absence of BFA, small amounts of radioactivity are associated with OSP-like material in the extracellular medium. However, due to the small amounts recovered, this material could not be characterized and it remains to be determined whether or not it actually corresponds to OSPs. Furthermore, the summed radioactivity associated with OSP-like material and nOS recovered from the medium of control cells was not sufficient to account for the amount of radioactivity associated with cellular OSPs recovered from BFA-treated cells. So, does BFA block further metabolism of normally generated OSPs? We cannot rule out this possibility; but in experiments with concanamycin A, the vacuolar proton pumping ATPase inhibitor, or the ionophore, monensin, which increase the pH of acidic organelles such as lysosomes, endosomes, and GA (61), we were unable to reproduce the effects of BFA on OSP metabolism (A. Massarweh et al., unpublished observations). Furthermore, NZ inhibits the BFA-promoted appearance of OSPs. If the only effect of BFA is to block the degradation of constitutively generated OSPs in the endomembrane system, then it has to be hypothesized that NZ relieves this blockade. Although this cannot be ruled out, it seems unlikely, as we demonstrate that NZ does not affect the capacity of BFA to block the secretory pathway.

The second explanation for the effect of BFA on OSPs is that this drug promotes an increase in OSP generation from mature DLO. For example, BFA could increase the expression of an OSP generating enzyme, or increase the access of an OSP generating enzyme to DLO within the endomembrane system. In this regard, although the recently described Co$_{2+}$-activated DLODP activity is probably not the only OSP-generating enzyme, its activity was found to be similar in both control and BFA-treated HepG2 cells (A. Massarweh et al., unpublished observations). BFA is known to provoke the unfolded protein response and it is possible that this leads to changes in expression of enzymes involved in OSP metabolism. However, for the 3 h BFA incubation times reported here, BFA did not activate the unfolded protein response, as judged by changes in either CNX or protein disulfide isomerase expression measured by Western blot (A. Massarweh et al., unpublished observations). BFA could also block the export from the ER of a newly synthesized DLODP whose normal subcellular localization is in a post ER compartment. In this situation DLO would be cleaved in a nonphysiological process. We can rule out this explanation because pre-treating cells with NZ before BFA treatment essentially causes an ER export block and, under these conditions, we show that there is actually a reduction in BFA-provoked OSPs. The recently described Co$_{2+}$-activated DLODP activity codistributes with the trans GA marker, UGT (see Fig. 1A), during density gradient fractionation of mouse liver microsomes (18). Here we show that these DLODP and UGT activities are associated with membranes of similar density in both the absence and presence of BFA, suggesting these two activities may colocalize in the GA. Interestingly, MAN2A1, which is considered to be a marker for the medial GA (see Fig. 1A), shows a greater BFA-induced shift on the density gradient than UGT or DLODP, suggesting that Co$_{2+}$-activated DLODP and MAN2A1 are differently distributed in HepG2 membranes. When HepG2 cells are treated with BFA, there is an increase in codistribution of membranes containing ER markers with those containing Co$_{2+}$-activated DLODP, suggesting that fusion of DLO-containing ER membranes with DLODP-containing GA membranes could underlie the increased OSPs seen in BFA-treated cells. To summarize, presently we do not know why BFA provokes an increase in OSPs derived from mature DLO in the lumen of the endomembrane system, but taking into account that this process is blocked by NZ and that subcellular fractionation studies demonstrate a BFA-provoked shift in the density of DLODP-containing membranes toward that of ER membranes, we cannot exclude the hypothesis that BFA causes the mixing of a GA-situated DLODP activity with ER-situated DLO.

**What is the physiological significance of OSP generation from mature DLO within the endomembrane system?**

If it is hypothesized that BFA merely blocks the catabolism of OSPs that are constitutively generated from mature DLO within the ER, then OSP generation in cells derived from ALG6-CDG and ALG8-CDG patients, which accumulate Glc$_{1-3}$Man$_2$GlcNAc$_2$-PP-Dol, may actually be much higher than previously noted (14, 15). In this situation OSP release may reflect a mechanism involved in reducing accumulation of unwanted DLO and recycling DolP. On the other hand, the physiological significance of the alternative mechanism, where translocation of a GA-situated DLODP into the ER underlies BFA-provoked OSP generation, remains obscure. The key to understanding this phenomenon may lie in finding out whether Golgi DLODP comes into contact with DLO under physiological conditions. For example, during mitosis, when GA enzymes associate with the ER (62), could Golgi DLODP play a role in DLO control? Alternatively, under other circumstances, DLO could flow out of the ER into the GA, and be hydrolyzed by DLODP.

To conclude, BFA causes MANEA1 processing of DLO so that the unusual (d$_2$,d$_3$)Man$_2$GlcNAc$_2$-PP-dolichol structure accumulates. This truncated DLO does not give rise to cytoplasmic Man$_2$GlcNAc$_2$-P, as has been shown for
smaller DLO species that accumulate in certain types of CDG. Nevertheless, BFA provoked a striking increase in OSFs recovered from within the endomembrane system without affecting the quantity of a cytoplasmic OSP derived from truncated DLO. Therefore the use of BFA has delineated at least two OSP-generating mechanisms, but further understanding of the compartmentalization and membrane topology of both DLO and DLODP activities will be required before insights into the function of OSPs recovered from within the endomembrane system will be available.

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REFERENCES

1. Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. 54: 631–656.
2. Burda, P., and M. Aebi. 1999. The dolichol pathway of N-linked glycosylation. Biochim. Biophys. Acta. 1426: 239–257.
3. Schenk, B., F. Fernandez, and C. J. Waechter. 2001. The (inside) and out(side) of dolichyl phosphate biosynthesis and recycling in the endoplasmic reticulum. Glycobiology. 11: 61R–70R.
4. Rush, J. S., N. Gao, M. A. Lehnman, and C. J. Waechter. 2008. Recycling of dolichyl monophosphate to the cytoplasmic leaflet of the endoplasmic reticulum after the cleavage of dolichyl phophoryl isoprenoid on the luminal monolayer. J. Biol. Chem. 283: 4087–4093.
5. Aebi, M., and T. Hennet. 2001. Congenital disorders of glycosylation (CDG-I).
6. Anumula, K. R., and R. G. Spiro. 1983. Release of glucose-containing polymannose oligosaccharides during glycoprotein biosynthesis. Studies with thyroid microsomal enzymes and slices. J. Biol. Chem. 258: 15274–15282.
7. Cacan, R., S. Duvet, D. Kmiecik, O. Labiau, A. M. Mir, and A. Verbert. 1998. 'Glyco-deglyco' processes during the synthesis of N-linked glycoproteins. Biochimie. 80: 54–59.
8. Cacan, R., and A. Verbert. 1997. Transfer of free polymannose-type oligosaccharides from the cytosol to lysosomes in cultured human hepatocellular carcinoma HepG2 cells. J. Cell Biol. 136: 45–59.
9. Moore, S. E., and R. G. Spiro. 1994. Intracellular compartmentalization and degradation of free polymannose oligosaccharides released during glycoprotein biosynthesis. J. Biol. Chem. 269: 12715–12721.
10. Fleischer, S., and M. Kervina. 1974. Subcellular fractionation of rat liver. Methods Enzymol. 31: 6–41.
11. Plonne, D., I. Cartwright, W. Lims, R. Dargel, J. M. Graham, and J. A. Higgins. 1999. Separation of the intracellular secretory compartment of rat liver and isolated rat hepatocytes in a single step using a two-point gradient of iodixanol. Anal. Biochem. 276: 88–96.
12. Doms, R. W., G. Russ, and P. Codogno. 1995. Endoplasmic reticulum-associated degradation (ERAD) and free oligosaccharide generation in Saccharomyces cerevisiae. J. Biol. Chem. 280: 41786–41800.
13. Durrant, C., and S. E. Moore. 2002. Perturbation of free oligosaccharide trafficking in endoplasmic reticulum glucosidase I-deficient and castanospermine-treated cells. Biochem. J. 365: 239–247.
14. Sugrue, T. F., R. I. Kohn, G. T. Hermanson, A. K. Milla, F. H. Garten, M. D. Provenzano, E. K. Fujimoto, N. M. Goekoe, B. J. Olson, and D. C. Klein. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 135: 50–55.
15. Wagner, R. R., and M. A. Cynkin. 1971. Glycopeptide metabolism: a UDP-galactose-glycoprotein galactosyltransferase of rat serum. Biochem. Biophys. Res. Commun. 45: 57–62.
16. Chandran, S., and C. E. Machamer. 2008. Acute perturbations in Golgi organization impact de novo sphingomyelin synthesis. Traffic. 9: 1894–1904.
17. Cruceanu, V., E. Leithé, and S. O. Mikalsen. 2003. Iliamquinone inhibits gap-junctional communication prior to Golgi fragmentation and block in protein transport. Exp. Cell Res. 287: 130–142.
18. Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H. P. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. Cell. 60: 821–836.
19. Sonoda, H., T. Okada, S. Jahanger, and S. Nakamura. 2007. Requirement of phospholipase D for ilimaquinone-induced Golgi membrane fragmentation. J. Biol. Chem. 282: 34085–34092.
20. Lippincott-Schwartz, J., L. C. Yuan, S. Bonifacino, and R. D. Klausner. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell. 58: 801–813.
21. Chavola, D., and R. C. Hughes. 1991. Effects of brefeldin A on oligosaccharide processing. Evidence for decreased branching of
complex-type glycans and increased formation of hybrid-type glycans. Biochem. J. 279: 159–165.

38. Sampath, D., A. Varki, and H. H. Freeze. 1992. The spectrum of incomplete N-linked oligosaccharides synthesized by endothelial cells in the presence of brefeldin A. J. Biol. Chem. 267: 4440–4455.

39. Misumi, Y., Y. Misumi, K. Miki, A. Takatsuji, G. Tamura, and Y. Ikehara. 1986. Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. J. Biol. Chem. 261: 11098–11103.

40. Siencz, J. B., W. J. Sun, J. W. Chang, J. Li, B. Bursulaya, N. S. Gray, and D. B. Haslam. 2009. Golgicide A reveals essential roles for GBF1 in Golgi assembly and function. Nat. Chem. Biol. 5: 157–165.

41. Lytton, J., M. Westlin, and M. R. Hanley. 1991. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. J. Biol. Chem. 266: 17067–17071.

42. Wong, W. L., M. A. Brostrom, G. Kuznetsov, D. Gmitter-Yellen, and C. O. Brostrom. 1993. Inhibition of protein synthesis and early protein processing by thapsigargin in cultured cells. Biochem. J. 289: 71–79.

43. Lubas, W. A., and R. G. Spiro. 1987. Golgi endo-alpha-D-mannosidase from rat liver, a novel N-linked carbohydrate unit processing enzyme. J. Biol. Chem. 262: 3775–3781.

44. Lubas, W. A., and R. G. Spiro. 1988. Evaluation of the role of rat liver Golgi endo-alpha-D-mannosidase in processing N-linked oligosaccharides. J. Biol. Chem. 263: 3990–3998.

45. Chege, N. W., and S. R. Pfeffer. 1991. Thapsigargin inhibits the Golgi complex: brefeldin-A distinguishes trans-Golgi cisternae from the trans-Golgi network. J. Cell Biol. 111: 893–899.

46. Torossi, T., J. Roth, and M. Ziak. 2007. A single tryptophan residue of endomannosidase is crucial for Golgi localization and in vivo activity. Cell. Mol. Life Sci. 64: 1881–1889.

47. Romero, F. A., and A. Herscovics. 1986. Transfer of monoglycosylated oligosaccharide from lipid to protein in a mammalian cell. J. Biol. Chem. 261: 15936–15940.

48. Chantret, L., T. Dupre, C. Delenda, S. Bucher, J. Danceourt, A. Barnier, A. Charollais, D. Heron, B. Bader-Meunier, O. Danos, et al. 2002. Congenital disorders of glycosylation type Iig is defined by a deficiency in dolichyl-P-mannose:Man7GlcNAc2-PP-dolichyl mannosyltransferase. J. Biol. Chem. 277: 25815–25822.

49. Dohi, K., J. Isoyama-Tanaka, R. Misaki, and K. Fujimaya. 2011. Jack bean alpha-mannosidase digestion profile of hybrid-type N-glycans: effect of reaction pH on substrate preference. Biochimie. 93: 766–771.

50. Cacan, R., S. Duvet, O. Labiau, A. Verbert, and S. S. Krag. 2001. Monoglycosylated oligomannosides are released during the deglycosylation process of newly synthesized glycoproteins. J. Biol. Chem. 276: 22297–22312.

51. Fouliquier, F., A. Harduin-Lepers, S. Duvet, I. Marchal, A. M. Mir, P. Delannoy, F. Chirat, and R. Cacan. 2002. The unfolded protein response in a dolichyl phosphate mannose-deficient Chinese hamster ovary cell line points out the key role of a demannosylation step in the quality-control mechanism of N-glycoproteins. Biochem. J. 362: 491–498.

52. Moore, S. E., and R. G. Spiro. 1993. Inhibition of glucose trimming by castanospermine results in rapid degradation of un assembled major histocompatibility complex class I molecules. J. Biol. Chem. 268: 3809–3812.

53. Strous, G. J., P. van Kerkhof, G. van Meers, S. Rijnboutt, and W. Stoovogel. 1993. Differential effects of brefeldin A on transport of secretory and lysosomal proteins. J. Biol. Chem. 268: 2841–2847.

54. Füllekrug, J., B. Sönnichsen, U. Schäfer, P. Nguyen Van, H. D. Söling, and G. Mieskes. 1997. Characterization of brefeldin A-induced vesicular structures containing cycling proteins of the intermediate compartment/cis-Golgi network. FEBS Lett. 404: 75–81.

55. Zuber, C., M. J. Spiro, B. Gahl, R. G. Spiro, and J. Roth. 2000. Golgi apparatus immunolocalization of endomannosidase suggests post-endoplasmic reticulum glucose trimming implications for quality control. Mol. Biol. Cell. 11: 4227–4240.

56. Casey, J. R., S. Grinstein, and J. Orlofski. 2010. Sensors and regulators of intracellular pH. Nat. Rev. Mol. Cell Biol. 11: 50–61.

57. Hassinen, A., A. Rivinoja, A. Kauppila, and S. Kellokumpu. 2010. Golgi N-glycosyltransferases form both homo- and heterodimeric enzyme complexes in live cells. J. Biol. Chem. 285: 17771–17777.

58. Rivinoja, A., A. Hassinen, N. Kokkonen, A. Kauppila, and S. Kellokumpu. 2009. Elevated Golgi pH impairs terminal N-glycosylation by inducing mislocalization of Golgi glycosyltransferases. J. Cell. Physiol. 220: 14–154.

59. Hassinen, A., and S. Kellokumpu. 2014. Organizational interplay of Golgi N-glycosyltransferases involves organelle microenvironment-dependent transitions between enzyme homo- and heteromers. J. Biol. Chem. 289: 26937–26948.

60. Sanyal, S., and A. K. Menon. 2009. Specific transbilayer translocation of dolichol-linked oligosaccharides by an endoplasmic reticulum flipase. Proc. Natl. Acad. Sci. USA. 106: 767–772.

61. Kim, J. H., C. A. Lingwood, D. B. Williams, W. Furuya, M. F. Manolson, and S. Grinstein. 1996. Dynamic measurement of the pH of the Golgi complex in living cells using retrograde transport of the verotoxin receptor. J. Cell Biol. 134: 1387–1399.

62. Sengupta, P., P. Satpute-Krishnan, A. Y. Sze, D. T. Burnette, G. H. Patterson, and J. Lippincott-Schwartz. 2015. ER trapping reveals Golgi enzymes continually revisit the ER through a recycling pathway that controls Golgi organization. Proc. Natl. Acad. Sci. USA. 112: E6752–E6761.