The Golgi apparatus: insights from lipid biochemistry

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Discovered by Camillo Golgi in 1898 [1, 2], the Golgi apparatus has long fascinated cytologists because of its distinct morphology [Fig. 1]. This organelle, which is found in all eukaryotic cells, consists of flat cisternae arranged in parallel to form the characteristic 'stacks' that are interconnected by cisternal or tubular-recticular bridges. In addition, numerous small membranous vesicles are usually found associated with the organelle. The Golgi apparatus is currently the focus of much attention among cell and membrane biologists because of its central role in directing protein traffic within cells. At least three areas, the cis, medial, and trans subsections can be seen by electron microscopy. The cis compartments are often situated closest to the nucleus, while the trans elements are most distant. From biochemical studies we know that there are at least three functionally distinct compartments that operate sequentially to build the oligosaccharide chains on transported glycoproteins and to 'sort' proteins destined for transport to other regions of the cell (e.g. the lysosomes, cell surface or secretory granules). Excellent reviews on various aspects of the Golgi apparatus, including its morphology and cytochemistry [3–5], biochemical organization [6, 7], and its roles in glycoprotein synthesis [8–10] and protein secretion and sorting [11–15], are available.

Until now, most biochemical and cell biological studies of the Golgi apparatus have concentrated on its role in the synthesis and sorting of proteins. In this paper I will highlight studies from my own laboratory dealing with some aspects of the metabolism and transport of lipids through the Golgi apparatus and indicate how such studies may enhance our understanding of the function of this organelle.

A vital stain for the Golgi apparatus

For a number of years, my colleagues and I have systematically studied the synthesis and transport of lipid molecules in animal cells. Our goal has been to define the pathways and molecular mechanisms by which the many different lipid species found in cells move from one organelle to another after their biosynthesis. This is a fundamental problem in membrane biology which has been difficult to study because simple techniques for examining lipid 'traffic' within cells are not available. We developed a new approach for studying this problem using a series of fluorescent lipid analogues in which one of the naturally occurring fatty acids is replaced by N-(4-nitrobenzo-2-oxa-1,3-diazole)amino-caproic acid \((\text{C}_n\text{-NBD-fatty acid})\) [16]. These lipids exhibit high rates of spontaneous transfer between membranes \textit{in vitro}, and this property permits us to readily integrate the fluorescent lipids into cellular membranes from exogenous sources. In our early studies, we found that when the various analogues were incubated with cells, they were metabolized similarly to their endogenous counterparts. Because the...
molecules were fluorescent, we were able to examine their distribution within living cells by high-resolution fluorescence microscopy and correlate changes in intracellular distribution with lipid metabolism (for a review, see [16]). The observed metabolism of these molecules was particularly significant because it demonstrated that even though these synthetic lipids were fluorescent and therefore 'non-natural', the enzymes involved in lipid metabolism recognized them.

Our interest in the Golgi apparatus began in 1981 when Naomi Lipsky, a former post-doctoral fellow, joined my laboratory. I suggested that she synthesize a fluorescent analogue of ceramide, [N-(E-NBD-aminocaproyl)-o-erythro-sphingosine; C$_{24}$-NBD-Cer; Fig. 2], and study its behavior in cells. This analogue was potentially interesting because endogenous ceramide is a key intermediate in sphingolipid biosynthesis — it is metabolized to sphingomyelin, a major structural lipid in cells, and to glycolipids and gangliosides, relatively minor lipids which play important roles in cell function (e.g., cell–cell recognition).

In our initial studies [17], using Chinese hamster fibroblasts, we found that when cells are treated with C$_{24}$-NBD-Cer at low temperature and washed, the endoplasmic reticulum (ER), nuclear envelope, and mitochondrion become fluorescently labelled. Upon warming the cells to 37°C, the fluorescent ceramide is metabolized to fluorescent sphingomyelin (SM) and glucosylceramide (GlcCer) and, concomitantly, the Golgi apparatus becomes intensely fluorescent. After additional time at 37°C, the plasma membrane also becomes visibly labelled as fluorescent SM and GlcCer are transported to the cell surface. Transport of the newly synthesized fluorescent sphingolipids to the plasma membrane is inhibited in mitotic cells [18] and by the ionophore monensin [19], as is the movement of newly synthesized glycoproteins to the cell surface under these conditions [20–22]. These results indicate that the fluorescent SM and GlcCer analogues are synthesized intracellularly and then transported from the Golgi apparatus by a vesicle-mediated process analogous to the movement of plasma membrane and secretory proteins [23] between the Golgi apparatus and the plasma membrane.

In a study published in 1985, we showed that C$_{24}$-NBD-Cer prominently stained the Golgi apparatus of a number of different cell types and was thus a useful stain for Golgi visualization [24]. Since then, we have used this fluorescent analogue on numerous cell types — in every case we have found prominent labelling of this unique organelle. To our knowledge, C$_{24}$-NBD-Cer is the only known vital stain for the Golgi apparatus.

As a result of these findings, many laboratories are now using C$_{24}$-NBD-Cer in various cell biological studies of the Golgi apparatus. One particularly interesting study [25] carried out by van Meer and colleagues demonstrated that in polarized epithelial MDCK (Madin-Darby canine kidney) cells, C$_{24}$-NBD-Cer is metabolized to C$_{24}$-NBD-SM and -GlcCer and these lipid metabolites are preferentially delivered to the apical cell surface [25]. This polarized delivery is consistent with the known enrichment of glycosphingolipids and SM, mainly Cer and GlcCer, in the apical membrane and secretory proteins.

Over the years we have attempted to define the aspects of the structure of C$_{24}$-NBD-Cer which are responsible for its unique properties described above. In one study [28], we synthesized a series of fluorescent N-acyl-sphingosine analogues, similar to C$_{24}$-NBD-Cer, using various long-chain bases and fluorescent fatty acids (Fig. 3) and then studied their metabolism and intracellular distribution in cultured fibroblasts. While most of the fluorescent N-acyl-sphingosines were significantly metabolized to the corresponding fluorescent SM, metabolism of the fluorescent GlcCer was strongly dependent on the long-chain base and the stereochemistry of the fluorescent fatty acid moiety. When cells were incubated with the various fluorescent N-acyl-sphingosine analogues under appropriate conditions, prominent labelling of the Golgi apparatus was seen in all cases except for N-(E-NBD-aminohexanoyl)-3-keto-sphingosine. These findings support the idea that other N-acyl-sphingosine derivatives might be useful in future studies of (glyco)sphingolipid metabolism, sorting, and transport. In particular, an N-acyl-sphingosine analogue which accumulates in the Golgi apparatus and which bears a radioactive and photoactivatable fatty acid might be useful in identifying some of the proteins in the Golgi apparatus which are involved in these processes.

Although a large number of membrane proteins would probably be labelled in such an experiment, the use of different long-chain bases, as described above, could modify the metabolism and intracellular distribution of the labelled N-acyl-sphingosine and in identifying the proteins of interest. [In preliminary experiments, we have synthesized N-erythro-sphingosines which are N-acylated with short-chain radioactive and/or photoactivatable fatty acids [3-4-hydroxy, 3-[125$I]$di-iodophenyl]propionic acid (Bolton and Hunter reagent), 3-[125$I]jodo, 4-azidosalicylic acid] or with a biotinylated fatty acid, and found them to be metabolized in a manner similar to C$_{24}$-NBD-Cer in cultured fibroblasts (R. E. Pagano, unpublished work).]
Recently, in collaboration with Drs Richard Haugland and Hee Chol Kang of Molecular Probes, Inc. (Eugene, OR, U.S.A.), we have begun to explore other ceramide analogues, similar to C<sub>27</sub>-NBD-Cer, in which different fluorochromes are substituted for the NBD moiety. Our hope is to obtain additional fluorescent analogues which are metabolized and transported in a manner similar to C<sub>27</sub>-NBD-Cer, but which fluoresce at different wavelengths. Such probes might then be used with C<sub>27</sub>-NBD-Cer in resonance energy transfer microscopy studies [29] to examine the kinetics of lipid translocation through the Golgi apparatus of single living cells.

The Golgi apparatus as a 'molecular trap' for ceramide [30, 31]

The labelling of the Golgi apparatus by C<sub>27</sub>-NBD-Cer is so highly specific and striking that we further investigated the mechanism responsible for its accumulation in that organelle. (Control experiments using other C<sub>27</sub>-NBD-lipids showed that Golgi labelling is specific for ceramide and is not simply due to the presence of the NBD fluorophore.) One possibility is that the fluorescent lipid, and perhaps its metabolites, are transported to the Golgi apparatus by an energy- and temperature-dependent process analogous to that seen for the delivery of newly synthesized proteins from the ER to the Golgi apparatus. Alternatively, C<sub>27</sub>-NBD-Cer labelling might be due to binding of the fluorescent lipid to enzymes involved in ceramide metabolism, or the lipid might preferentially partition into this organelle as a result of some special 'physical property' of the Golgi membranes. In our initial attempts to distinguish between these possibilities, we were unable to inhibit C<sub>27</sub>-NBD-Cer labelling of the Golgi apparatus at low temperatures or in the presence of various metabolic inhibitors.

Surprisingly, labelling of the Golgi apparatus even occurred in cells which had been fixed before incubation with the fluorescent lipid. When cells are fixed with glutaraldehyde and subsequently incubated with C<sub>27</sub>-NBD-Cer, labelling of the Golgi apparatus is detected, but is difficult to see clearly because of a high 'background' from other fluorescently labelled intracellular membranes within the treated cells. However, this background fluorescence can be removed when the labelled cells are further incubated ('back-exchanged') with defatted albumin. The result is a preparation of fixed cells in which only the Golgi apparatus is prominently stained by C<sub>27</sub>-NBD-Cer (Fig. 4).

We also developed a method for visualizing the fluorescent lipid at the level of the electron microscope, based on the photoconversion of a fluorescent marker to a diamino benzidine (DAB) product [32, 33]. In this procedure, cells labelled with C<sub>27</sub>-NBD-Cer are irradiated in the presence of DAB. During irradiation, the fluorophore is photobleached and the photo-oxidation products catalyse the polymerization of DAB. This results in an electron-opaque osmiophilic polymer which appears black when visualised in the electron microscope and is present at sites within the specimen which were originally labelled by the fluorescent lipid [31]. As shown in Fig. 5, this procedure localizes C<sub>27</sub>-NBD-Cer to only one or two stacks of the Golgi apparatus, although numerous small vesicles were also labelled in some areas, possibly resulting from tangential sections through the Golgi cisternae. Using other markers for known subcompartments of the Golgi apparatus in double-label experiments, we determined that C<sub>27</sub>-NBD-Cer labelling is restricted to the trans-Golgi cisternae [31].

Several pieces of evidence suggest that the trapping of C<sub>27</sub>-NBD-Cer at the Golgi apparatus of fixed cells is due to its

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The table and diagram are included in the text as follows:

### Table: Long-chain bases and Fluorescent fatty acids

<table>
<thead>
<tr>
<th>Long-chain bases</th>
<th>Fluorescent fatty acids</th>
</tr>
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<tbody>
<tr>
<td>Sphingosine</td>
<td>α-NBD-aminohexanoic acid</td>
</tr>
<tr>
<td>Dihydrosphingosine</td>
<td>α-OH, α-NBD-aminohexanoic acid</td>
</tr>
<tr>
<td>Phytosphingosine</td>
<td>α-NBD-aminohexanoic acid</td>
</tr>
<tr>
<td>3-Ketosphingosine</td>
<td>α-NBD-aminohexanoic acid</td>
</tr>
</tbody>
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Asterisks indicate asymmetric carbon atoms which result in stereoisomers of the indicated compounds. The following stereoisomers were used in the synthesis of the fluorescent N-acyl-sphingosines [28]: α-erythro-sphingosine, α-threo-sphingosine, α-threo-dihydrosphingosine, α- or l-α-OH, α-NBD-aminohexanoic acids, α- or l-α-NBD-aminohexanoic acids.

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**Fig. 3. Various long-chain bases and fluorescent fatty acids used in the synthesis of fluorescent compounds structurally related to C<sub>27</sub>-NBD-Cer**

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**Fig. 4.** Visualization of the fluorescent lipid at the level of the electron microscope, based on the photoconversion of a fluorescent marker to a diamino benzidine (DAB) product [32, 33]. In this procedure, cells labelled with C<sub>27</sub>-NBD-Cer are irradiated in the presence of DAB. During irradiation, the fluorophore is photobleached and the photo-oxidation products catalyse the polymerization of DAB. This results in an electron-opaque osmiophilic polymer which appears black when visualised in the electron microscope and is present at sites within the specimen which were originally labelled by the fluorescent lipid [31]. As shown in Fig. 5, this procedure localizes C<sub>27</sub>-NBD-Cer to only one or two stacks of the Golgi apparatus, although numerous small vesicles were also labelled in some areas, possibly resulting from tangential sections through the Golgi cisternae. Using other markers for known subcompartments of the Golgi apparatus in double-label experiments, we determined that C<sub>27</sub>-NBD-Cer labelling is restricted to the trans-Golgi cisternae [31].

Several pieces of evidence suggest that the trapping of C<sub>27</sub>-NBD-Cer at the Golgi apparatus of fixed cells is due to its...
interaction with endogenous Golgi apparatus lipids. First, accumulation of $C_{16}$-NBD-Cer occurs in cells fixed in various ways, but this accumulation is inhibited when fixation protocols which extract or modify cellular lipids are used. For example, glutaraldehyde-fixed cells rendered permeable by brief treatment with detergents and then washed extensively do not exhibit post-fixation staining of the Golgi apparatus by $C_{16}$-NBD-Cer, although such treatments have no obvious effect on the morphology of the Golgi apparatus as determined with fluorescent antibodies at the light microscope level. Secondly, the amount of $C_{16}$-NBD-Cer present after back-exchange indicates that $1.0\times10^8$ molecules of the fluorescent lipid are present at the labelled Golgi cisternae of each cell. Therefore, $C_{16}$-NBD-Cer labelling of the Golgi apparatus is probably not due to its binding to a resident Golgi apparatus protein(s), since the required number of copies of this protein(s) would be extraordinarily high. Finally, Filipin, a fluorescent polyene antibiotic which forms complexes with cellular cholesterol and labels the Golgi apparatus of fixed cells [34, 35], inhibits accumulation of $C_{16}$-NBD-Cer at that organelle.

Recently, in collaboration with Drs Joan Blanchette-Mackie and Peter Pentchev of the U.S. National Institutes of Health, we have begun to explore the effects of alteration of cellular cholesterol on accumulation of $C_{16}$-NBD-Cer at the Golgi apparatus. We found that when cellular cholesterol was depleted, either by growth of cells in lipoprotein-deficient serum or in medium containing inhibitors of cholesterol biosynthesis, $C_{16}$-NBD-Cer labelling of the Golgi apparatus was dramatically reduced (R. E. Pagano, O. C. Martin, M. E. Comly, J. Blanchette-Mackie & P. Pentchev, unpublished work). Golgi labelling could be restored by stimulating endogenous cholesterol biosynthesis using mevalonic acid, a precursor of cholesterol biosynthesis, or by supplying exogenous cholesterol to the growth medium. These striking observations suggest that the sterol content of the trans-Golgi cisternae plays a critical role in $C_{16}$-NBD-Cer labelling. However, since cholesterol is also present in other cellular membranes to which $C_{16}$-NBD-Cer does not localize, we speculate that additional Golgi apparatus constituents, perhaps the endogenous (glyco)sphingolipids found there,
Sphingomyelin synthesis occurs predominantly at the cis and medial cisternae of the Golgi apparatus

Our studies with C$_2$-NBD-Cer in living cells [17, 19] suggested that the Golgi apparatus was the major site of SM synthesis. However, this conclusion was somewhat controversial since previous studies from other laboratories suggested that the ER, plasma membrane, Golgi apparatus, or some combination of these, was the site of SM synthesis. On the other hand, the biochemical pathway leading to SM synthesis is well characterized [36]. SM is synthesized in mammalian tissues from ceramide by the transfer of phosphorylcholine directly from phosphatidylcholine (PC):

\[
\text{PC} + \text{ceramide} \rightarrow \text{SM} + \text{DAG}
\]

generating a molecule of diacylglycerol (DAG) for each molecule of SM formed.

Given the formation of DAG, an important second messenger in cells [37], and the possible implications for intracellular lipid movement if SM synthesis were to take place at the Golgi apparatus [38], we decided to determine rigorously the intracellular site of SM synthesis [38a]. This work was performed by Anthony Futerman, a post-doctoral fellow in the laboratory, in collaboration with Drs Ann Hubbard and Bruno Steiger of the Johns Hopkins School of Medicine. Well-characterized subcellular fractions were isolated from rat liver, incubated with a radiolabeled analogue of ceramide, N-[1-14C]hexanoyl-L-erythro-sphingosine (Fig. 2), and the synthesis of radioactive SM was quantified. Using a Golgi apparatus fraction which was highly enriched in the marker enzyme, galactosyl transferase, and which showed very little cross-contamination with other subcellular compartments, we found a 85–98-fold enrichment of SM synthase activity. Small amounts of SM synthase activity were also associated with enriched plasma membrane and rough microsome fractions, but, after accounting for the contamination of these fractions by Golgi apparatus membranes, together these fractions contributed less than 13% of the total SM synthase activity in liver. We conclude that at least 87% of the SM synthesized in rat liver occurs at the Golgi apparatus.

We have further separated the membranes of the Golgi apparatus into fractions of heavy, intermediate, and light density using the procedure of Ehrenreich et al. [39]. These various fractions result from the maturation of nascent lipoproteins as they migrate through the Golgi complex and acquire lipid, becoming lighter in density. As a result, Golgi fractions from trans cisternae are lighter in density than those obtained from the cis and medial cisternae. These subfractions of the Golgi apparatus were definitively characterized by immunoblotting and biochemical assays using cis/medial (mannosidase II) and trans (sialyl transferase and galactosyl transferase) Golgi apparatus markers. Cis and medial fractions were enriched in SM synthesis, with far less activity associated with a trans fraction. Thus we conclude that SM synthesis occurs predominantly in the cis and medial cisternae of the Golgi apparatus. This conclusion is supported by our earlier studies using C$_2$-NBD-Cer in cultured fibroblasts in which it was found that the ionophore monensin did not interfere with the synthesis of C$_2$-NBD-SM from C$_2$-NBD-Cer, but did inhibit the transport of the newly synthesized SM to the plasma membrane [19]. Since monensin blocks medial to trans movement of glycoproteins [40, 41], this result suggested that SM synthesis occurred in early compartments of the Golgi apparatus.

Our studies demonstrating that C$_2$-NBD-Cer preferentially labels the trans-Golgi stacks of (fixed) cells [31], while metabolism to SM occurs in the cis and medial-Golgi elements [38a], presents us with a paradox since the lipid substrate is delivered distal to the compartment(s) where it is metabolized. One possible explanation for this may be that ceramide undergoes retrograde transport within the Golgi apparatus during SM synthesis. Alternatively, there may be multiple pools of C$_2$-NBD-Cer in living cells which could account for these findings.

ATP-dependent fusion of liposomes with the Golgi apparatus [42]

Lastly, I want to mention a remarkable finding concerning the Golgi apparatus which was made by Toshihide Kobayashi when he was a post-doctoral fellow in this laboratory. For years I had been interested in microinjecting fluorescently-tagged lipid vesicles (liposomes) into the cytoplasm of cells to determine whether such vesicles would preferentially associate with certain organelles. If so, it would then be possible to systematically evaluate the roles of vesicle size, lipid composition, charge, and other parameters on the distribution of these vesicles within cells. Unfortunately, such experiments were not technically possible because, invariably, the lipid vesicles clogged the micropipettes used for introducing them into cells. However, about the time Dr Kobayashi joined the laboratory, several new methods were introduced for generating semi-intact or ‘perforated’ cells [43, 44]. Importantly, these preparations were shown to support endogenous vesicle transport and fusion. With the perforated-cell system we were able to readily introduce artificial lipid vesicles, labelled with various fluorescent markers, into cells and examine their interactions with intracellular membranes [42]. To our surprise, we found that when incubations were performed in the presence of an ATP-regenerating system, both vesicle lipids and entrapped water-soluble markers were transferred to the Golgi apparatus of treated cells, indicative of fusion of the liposomes with the membranes of the Golgi apparatus. Fusion occurred using unilamellar vesicles 30–80 nm in diameter and comprised of phosphatidylincholine, but was inhibited by pretreatment of the cells with N-ethylmaleimide. These findings demonstrated that lipid vesicles could be used to deliver labelled lipids, macromolecules, and dyes to the Golgi apparatus, and suggested several possibilities for future study. Experiments by Peter Hoffmann-Bleihauer, another post-doctoral fellow working on this system, are currently in progress to determine whether (i) liposomes fuse with all stacks of the Golgi apparatus, or only a subset, and (ii) labelled lipids, once delivered to the Golgi apparatus, are capable of ‘recycling’ back to the ER of the treated cells.

Future directions

Our studies with fluorescent and radioactive analogues of ceramide have yielded a vital stain for the Golgi apparatus and established this organelle as the major site for sphingomyelin biosynthesis. It is my hope that future studies will provide additional insights on how lipid and protein sorting and translocation through the Golgi apparatus are interrelated and controlled. In particular we hope to (i) examine the interrelationship of cholesterol biosynthesis and sphingolipid metabolism in cells and determine the molecular signal(s) for ceramide trapping at the Golgi apparatus; (ii) determine whether retrograde transport of ceramide within the Golgi apparatus occurs; (iii) identify and isolate the Golgi apparatus enzyme responsible for SM synthesis; and (iv) determine whether ‘recycling’ of lipids from the Golgi apparatus to the ER occurs.
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