Endocytic Membrane Traffic to the Golgi Apparatus in a Regulated Secretory Cell Line*

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We have established a ricin-resistant glycosylation-defective PC12 pheochromocytoma cell line to study biochemically glycoprotein traffic from the cell surface to the Golgi apparatus in regulated secretory cells. The strategy employed in this study is a modification of that used previously (Duncan, J. R., and Kornfeld, S. (1988) J. Cell Biol. 106, 617–628) to demonstrate transport of the cation-independent and -dependent mannose 6-phosphate receptors from the cell surface to the trans-Golgi network in nonsecretory cell types. In ricin-resistant PC12 cells, radiolabeled galactose was incorporated enzymatically into surface glycoconjugates, primarily glycoproteins. Resistance to β-galactosidase was acquired upon reculture at 37 °C due to further terminal glycosylation of the galactose residues. Treatment of N-linked oligosaccharides isolated from recultured cells with a variety of glycosidases in conjunction with β-galactosidase demonstrated the addition of sialic acid N-acetylglucosamine and fucose residues to the galactose residues in recultured cells. Resistance to β-galactosidase was not acquired in cells recultured at 19 °C, indicating that subsequent glycosylation of galactose residues did not occur at the cell surface or in endosomes. While glycosylation of galactose incorporated into asparagine oligosaccharides in Chinese hamster ovary clone 13 cells was not significant (<1%) after 6 h of reculture, approximately 10% of the galactose incorporated into surface oligosaccharides was further glycosylated in PC12 cells in this time. Analysis of total labeled versus β-galactosidase-resistant proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that endocytic traffic to the site of glycosylation activity in mutant PC12 cells was highly selective, but included a much greater number of proteins than were detected in Chinese hamster ovary clone 13 fibroblasts.

Since the initial demonstration by Snider and Rogers (1) that the transferrin receptor can be transported from the cell surface to the Golgi apparatus, it has been established using a variety of methods that both the cation-dependent and the -independent mannose 6-phosphate receptors can follow the same endocytic route. These proteins are transported from the cell surface to the trans Golgi network (TGN), the site of sialyltransferase activity (2), with half times of 1–4 h (1, 3, 4, 5). In contrast, bulk transport of plasma membrane glycoproteins (3) or glycoconjugates (5) to the TGN is extremely inefficient. Similarly, in the epithelial MDCK cell line, only a few proteins recycle efficiently between the apical plasma membrane and the TGN (6), while the cation-independent mannose 6-phosphate receptor cycles with relatively high efficiency between the basolateral plasma membrane and the TGN (7). Thus, in the cell types examined, glycoprotein transport from the plasma membrane to the TGN is highly selective, and proceeds at rates that are much slower than the recycling of receptors between the cell surface and endosomes (8).

In secretory cells, which have a large number of vesicles devoted to transporting secretory products from the Golgi apparatus to the plasma membrane, vesicle membrane must be rapidly internalized from the cell surface following exocytosis in order to maintain the steady state distribution of membrane area between the compartments (9). There is biochemical evidence that at least some of that membrane is recycled to the Golgi apparatus (10, 11). This is presumed to be true in cells exhibiting constitutive secretion, in which the Golgi-derived secretory vesicles contain the secretory cargo fuse with the plasma membrane soon after they are made, as well as in regulated secretory cells, in which the Golgi-derived vesicles (granules) accumulate in the cytoplasm until an external stimulus triggers rapid exocytosis (12, 13). Morphological studies documenting the transport of bulk membrane or fluid phase tracers to the Golgi apparatus in secretory cells indicate that traffic from the cell surface to the Golgi apparatus is a significant pathway in these specialized cell types (12, 14–17). Bulk endocytic tracers cannot readily be traced to the Golgi apparatus in cells that are not specialized for secretion (18–20) (which for simplicity we will refer to as “nonsecretory” cells), presumably because these cells have relatively less membrane and fluid flow through this pathway. These results suggest that secretory cells transport more fluid and membrane area from the cell surface to the Golgi apparatus than nonsecretory cell types, perhaps as a consequence of having to recycle secretory vesicle membrane components.

Since morphological studies of endocytosis to the Golgi apparatus have relied primarily on nonspecific fluid and membrane tracers and/or detection of enzymatic reaction products, quantitative analysis of this pathway, and identification of its components, has been limited. The biochemical methods used to study the transport of transferrin receptors and mannose

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The abbreviations used in this paper are: TGN, trans Golgi network; BSA, bovine serum albumin; BSII, Bandeiraea simplicifolia lectin II; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; CHO, Chinese hamster ovary; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholineethanesulfonic acid.
6-phosphate receptors can be used to obtain quantitative information. Using cell lines that are deficient in the addition of a specific sugar residue, or by experimentally modifying exposed oligosaccharides on the cell surface, substrates for Golgi glycosyltransferases can be created and radioiodinated at the cell surface. The subsequent activity of the Golgi glycosyltransferases can be created and radioiodinated at the cell surface. The subsequent activity of the Golgi enzymes on these proteins can be monitored quantitatively. These methods have thus far been applied only to nonsecretory cell types.

In order to develop a system to study quantitatively the traffic of cell surface glycoproteins to the Golgi apparatus in regulated secretory cells, we have selected glycosylation-defective mutants of PC12 pheochromocytoma cells for growth in the presence of a galactose-binding toxin, ricin. Here we describe the properties of the ricin-resistant PC12 cells, and our use of these cells to measure glycoprotein transport from the cell surface to the Golgi apparatus. We found that in PC12 cells, as in nonsecretory cell types, transport of cell surface proteins to the Golgi apparatus is highly selective. In contrast to the nonsecretory cell types, transport from the cell surface to the Golgi apparatus in PC12 cells appears to represent a major endocytic pathway for a greater number of glycoproteins.

MATERIALS AND METHODS

Chemicals—Except where noted, chemicals were purchased from Sigma.

Enzymes—Flavobacterium meningosepticum peptide N-glycosidase F, Diplococcus pneumoniae β-galactosidase and β-N-acetylgalactosaminidase, bovine kidney α-fucosidase, Bacteroides fragilis endo-β-galactosidase, Heliix pomatia arylsulfatase and Vibrio cholerae neuraminidase were purchased from Boehringer Mannheim. Bovine milk galactosyltransferase and Clostridium perfringens neuraminidase were from Sigma.

Cells—PC12 cells were grown in Dulbecco's minimal essential medium (Cell Culture Facility, University of California, San Francisco) containing 5% fetal bovine serum (HyClone Laboratories, Logan, UT) and 10% horse serum (HyClone Laboratories), and were passaged by trituration in divalent cation-free phosphate buffered saline (PBS) containing 10 mM Hepes, pH 7.2, and 1 mM EDTA. Chinese hamster ovary (CHO) clone 13 cells (21), kindly provided by Dr. Stuart Kornfeld (Washington University, St. Louis, MO), were grown in α-modified Eagle's medium containing 10% fetal bovine serum (HyClone Laboratories), and were passaged without trituration in cold acetate-free phosphate buffered saline containing 10 mM Hepes, pH 7.2.

Selection of Ricin-resistant Cells—PC12 cells were grown in medium containing various concentrations of Ricin communis toxin (RCαm, Sigma). 10 ng/ml ricin toxin was sufficient to kill >99% of the cells within 4 days. For selection of ricin-resistant clones, 5 x 10^5 cells were plated in each of three 10-cm dishes and fed with medium containing 10 ng/ml ricin. After 4 days, at which time most of the cells had died, cells were refed with 20% PC12-conditioned medium containing ricin. Clones were visible after 5 days, and were passaged using cloning rings after 19 days. Large stocks of selected clones were grown up without ricin for storage in liquid nitrogen. After the initial freezing, clones were passed not more than 5 times, and were generally resuspended after thawing for one passage (3 days) in 20 ng/ml ricin. Selection was performed on mutagenized PC12 cells, however all colonies were subsequently passaged using cloning rings after 19 days.

Lectin Binding Assays—Ricin toxin (RCαm) and Bandeiraea simplicifolia lectin II (BSII) (Sigma) were iodinated to low specific activity using iodobead. Free iodine was removed by ion-exchange chromatography. The activity of the ricin-resistant mutants of PC12 pheochromocytoma cells for growth in the presence of 0.5 mCi of Na2125I (Amersham Corp.) per sample and twice the published concentration of UDP-galactose (Sigma) and ATP. Sialytransferase was measured as described (23) using 1.2 μCi of CMP-[3H]neuraminic acid (Du Pont-New England Nuclear) per sample with a final concentration of 0.18 mM CMP-neuraminic acid (Sigma). For both assays, samples were precipitated for 10 min with ice-cold 20% trichloroacetic acid, 1% phenol-phosphotungstic acid, 0.5 M HCl, pelleted by centrifugation at 16,000 x g for 10 min, and washed once with the acid mixture. The pellets were solubilized in 300 μl of NCS tissue solubilizer (Amersham Corp.), neutralized with 15 μl of glacial acetic acid, and counted in 4 ml of Ekolume scintillation solution (ICN/Schwarz/Mann). Protein was quantitated by dye binding (24) using BSA as a standard.

Uptake and Release of Norepinephrine from PC12 Cells—The conditions for loading PC12 cells and the glycosylation mutants with [3H]norepinephrine (Amersham Corp.), in the presence of pargyline and stimulation of exocytosis by membrane depolarization with 15 mM KCl in the presence of carbamylcholine chloride were exactly as described (23). In this study, cells were washed twice after uptake of labeled norepinephrine and incubated for 8 min in 1 ml of culture medium at 37°C. This medium was collected, and an additional 1 ml of medium with or without added KCl (55 mM final concentration) and 5 mM carbamylcholine chloride was added to each dish. The final incubation medium was collected after 8 min, and 100-μl aliquots of all culture media were counted in 4 ml of Aquasol (New England Nuclear).

Exogalactosylation—Exogalactosylation of cells was carried out as described (3, 23, 28). Cells on polylysine-coated 3.5-cm or 6-cm tissue culture plates were labeled at 75–90% confluence, usually 1–2 (PC12 cells) or 2–3 days (CHO clone 15 cells) after plating. Cells were cooled to 0°C and washed twice with ice-cold exogalactosylation buffer (23) containing 0.25 ml per 3.5-cm dish or 0.5 ml per 6-cm dish for 45–50°C. Cells were then labeled with 5 x 10^6 cells were resuspended in 500 μl of cold PBS containing 1 mg/ml glucose.

Lipid Extraction—PC12 A1 cells exogen-derivatized with [3H]galactose were washed four times with PBS, and then harvested in 5% trichloroacetic acid. Cells were pelleted at 12,000 x g for 10 min, and the pellet washed three times with 200 μl of distilled water and disrupted by passage through a 25-gauge needle. The homogenate was aliquoted into three 180-μl samples and extracted according to the method of Dahms and Sehnhar (27). Methanol was added followed by chloroform to a chloroform:methanol:water ratio of 2:1:4. After vigorous mixing the extract was centrifuged at 12,000 x g for 10 min, and the phases were separated without solvent evaporation. The pellet was resuspended with 150 μl of solvent mixture. The remaining insoluble material was designated the protein fraction. The supernatants were pooled and water was added to a final solvent ratio of 4:8:5:6 (chloroform:methanol:water). The mixture was vortexed vigorously and the aqueous and organic phases separated by centrifugation at 12,000 x g for 10 min. Both phases were evaporated to dryness without solvent evaporation. The phases were prepared in parallel. The pooled aqueous and organic phases were dried under vacuum at 40°C. Organic phase material was dissolved in scintillation fluid and counted, and was found to contain <0.02% of the total radioactivity recovered in the extraction. Aqueous phase...
material, which is expected to include glycolipids, was resuspended in 600 ml of distilled water. To mimic the composition of the detergent lysates used in this study (see below), a 400-ml portion was adjusted to 0.15% Nonidet P-40; 50 mM NaCl in 100 ml of bovine serum albumin, and the sample was acid precipitated with an equal volume of 20% trichloroacetic acid, 1% phosphotungstic acid, 0.5 M HCl on ice for 10 min. The 16,000 × g pellet from this precipitation was washed with the resulting supernatant and the supernatant was dissolved in 40 ml of 8 M urea, 2% SDS, 20 mM diethiothreitol, 5 mM EDTA in a boiling water bath. These and the remaining 200 μl of the dissolved aqueous phase material were counted in 4 ml of scintillation fluid.

Detection of Terminally Glycosylated Proteins by Acid Precipitation—Radioactively exogalactosylated cells were lysed on the plates by the addition of 100 μl of lysis buffer I (0.5% Nonidet P-40, 50 mM sodium phosphate, pH 6.2) containing freshly added proteinase inhibitor cocktail (added from a 1000 × stock containing 10 μg/ml each of peptatin, chymostatin, leupeptin, and aprotonin in dimethyl sulfoxide and a 200 × stock containing 200 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, and 200 μg/ml o-phenanthroline in ethanol). The dishes were incubated 5 min on ice, the lysate collected, and 6-cm plates washed with an additional 100 μl of lysis buffer. Lysates were centrifuged at 30,000 × g for 15 min to remove nuclei and insoluble material. Portions of the supernatant were incubated for 14–16 h at 37 °C with or without the addition of 25–50 units of β-galactosidase. An additional 10–15 minutes of enzyme was then added, and the incubation continued for 2 h. Samples were precipitated with ice-cold 20% trichloroacetic acid, 1% phosphotungstic acid in 0.5 M HCl, and then washed with ice-cold acetone. The second addition of β-galactosidase was necessary to reduce the background, which was defined as acid precipitable counts resistant to digestion from cells that were lysed immediately after labeling, below 2% of the counts incorporated. In preliminary experiments, the precipitates were solubilized in 300 μl of NCS tissue solubilizer, neutralized with 15 μl of glacial acetic acid, and counted in 4 ml of scintillation fluid. For subsequent experiments precipitates were dissolved by heating in a boiling water bath in 40–80 μl of 8 M urea, 2% SDS, 20 mM diethiothreitol, 5 mM EDTA. For experiments that included analysis by SDS-polyacrylamide gel electrophoresis, the precipitates were resolubilized by sequential addition of 20–50 μl of 7 M urea, 20 mM diethiothreitol, followed by an equal volume of 2× concentrated electrophoresis sample buffer (28) and finally sample buffer to a final concentration of 4 M urea and 2% SDS and 1% β-mercaptoethanol and heated in a boiling water bath for 10 min. After cooling to room temperature, 10 milliliters of peptide-N-glycosidase F (29) was added, and the lysates were incubated for 15–20 h at 37 °C. The samples were then cooled to 0 °C, precipitated with 100 μl of 20% trichloroacetic acid, 1% phosphotungstic acid, 0.5 M HCl for 5 min, and centrifuged at 16,000 × g for 10 min at 4 °C. To minimize loss of α-linked sugar residues by acid hydrolysis, the supernatants were immediately adjusted to pH 8–9 with 20 μl of 1 N NaOH and 1.5 μl of saturated aqueous Tris base, and 1 μl of 300 mM Tris, pH 7.5, 2× gel filtration chromatography on a 0.6–22% seabed Cephadex G-25 (Pharmacia LKB Biotechnology Inc.) column. Radioactive fractions were pooled, dried under vacuum, and dissolved in 150–200 μl of 50 mM N-morpholinoethanesulfonic acid (MES) pH 6.0. Redissolved oligosaccharides (approximately 2 × 10^8 cpm in 40 μl) were incubated for 14–16 h at 37 °C with 10 milliliters of β-galactosidase. An additional 5 milliliters of β-galactosidase and the incubation continued for 2 h. Samples were then boiled for 3 min to inactivate the enzyme, made 10–20% (w/v) sucrose, and analyzed by gel filtration chromatography on Sephadex G-25. 350-μl fractions were counted in 4 ml of scintillation fluid.

To analyze terminal additions to galactose residues in recultured cells, some of the following glycosidases were added to oligosaccharide samples, either singly or in combination, in addition to 10 milliliters of β-galactosidase; 8–10 milliliters each of Vibrio cholerae and Clostridium perfringens neuraminidase, 8 milliliters of β-N-acetylgalactosaminidase, 15 μg of α-fucosidase, 8–10 milliliters of endo-β-galactosidase, 40 μg of arylsulfatase. Digestions were carried out in parallel with β-galactosidase digestions, and half of the original amount of each enzyme was added after 14–16 h. In some experiments portions of deactylated oligosaccharides were dried and dissolved in 10 μl of 2 M acetic acid, incubated for 60 min in a boiling water bath, neutralized with NaOH, and diluted into 80 μl of 50 mM MES prior to gel filtration treatment.

Electrophoresis and Fluorography—Sodium dodecyl sulfate (SDS)-polyacrylamide gels were run as described. After fixing in 10% methanol, 5% acetic acid, gels were rinsed with water and impregnated with 0.5 M sodium salicylate according to the method of Chamberlain (30). The dried gels were exposed to preflashed Kodak XAR5 film at −70 °C.

RESULTS

Experimental Strategy—One approach that has been used to study endocytic traffic to the Golgi apparatus entails selecting cell lines that are deficient in addition of terminal galactose residues to N-acetylgalactosamine residues on nascent glycoproteins. Glycoproteins synthesized in cells with such a defect do not incorporate sialic acid or other terminal sugar residues into their glycoproteins since the normal substrate for these additions, galactose, is not incorporated. This phenotype allows the enzymatic addition of radiolabeled galactose to the exposed cell surface glycoproteins followed by incubation of these cells to allow recycling to the Golgi apparatus. If glycoproteins radiolabeled in this way are recycled to the Golgi apparatus, they will be substrates for glycosyltransferases that normally act on terminal galactose residues, which were not incorporated when the proteins were synthesized. Addition of sugar residues to a radiolabeled galactose residue will render that galactose residue resistant to digestion with β-galactosidase, since this enzyme removes only terminal galactose residues from complex oligosaccharides. (3, 26).

Quantitation of β-galactosidase-resistant galactose residues on glycoproteins can then be accomplished either by analysis of isolated oligosaccharides by treatment with glycosidase and gel filtration chromatography (3), or by acid precipitation of glycosidase-treated glycoproteins. The strategy is summarized in Fig. 1.

Selection of Ricin-resistant PC12 Cells—To select PC12 cells with defective galactosylation of glycoproteins, cells were grown in the presence of ricin toxin. Ricin toxin binds to terminal galactose residues through its B chain, which normally facilitates the efficient internalization of the toxin (32). It was found that 10 ng/ml toxin killed >99% of wild type PC12 cells within 4 days of culture. This concentration of toxin was used to select PC12 cells from two 10-cm dishes (approximately 1 × 10^7 cells per dish). Nine clones were obtained from these plates, seven of which were extensively characterized.

Ricin-resistant PC12 Cells Exhibited Lecin Binding Properties Consistent with Defective Galactosylation—Ricin-resistant clones were initially screened for their capacity to bind radiolabeled ricin toxin. Of seven clones characterized, three bound >20% as much ricin per cell as wild type PC12 cells. The remaining four bound 13–16% as much as wild type cells (not shown). A more detailed analysis of lectin binding was performed on two of these four clones, A1 and A3. These cells were analyzed for their glycosylation phenotype by testing for binding of both ricin toxin (galactose specific) and Bandiera simplicifolia lectin II (BSII), a lectin specific for terminal N-acetylgalactosamine residues (33), either with or without prior exogalactosylation with unlabeled UDP-galactose (see "Materials and Methods"). Both clones bound approximately 6 times less ricin toxin and 16 times more BSII than wild type PC12 cells (Table I). Prior exogalactosylation increased ricin
binding above wild type levels and reduced BSII binding to approximately one quarter of control levels in both clones. Lectin binding was virtually abolished in the presence of the appropriate competing sugar (Table I). These studies demonstrate the presence of many more terminal N-acetylglucosamine residues, and many fewer galactose residues, on the cell surface glycoproteins of the PC12 A1 and A3 cells than on wild type PC12 cells, and that the selected clones cells are good substrates for the enzymatic addition of galactose.

CHO clone 13 cells, previously used for detection of glycoprotein traffic from cell surface to the TGN (3), were used as a representative nonsecretory cell type throughout this study. These cells are deficient in the ability to transport UDP-galactose into the lumen of the Golgi apparatus from the cytoplasm, resulting in the synthesis of glycoproteins with terminal N-acetylglucosamine residues (21, 34). Wild type PC12 cells and PC12 A1 cells were compared with CHO clone 13 cells for their capacity to bind ricin toxin and BSII lectin. Washed cells were incubated in suspension with various concentrations of radiolabeled lectin at 0 °C for 2 h prior to washing and counting. The lectin binding profiles of PC12 A1 cells and CHO clone 13 cells were similar for both ricin toxin (Fig. 2A) and BSII (Fig. 2B). As expected, wild type PC12 cells bound significantly more ricin toxin and less BSII than PC12 A1 cells or CHO clone 13 cells.

Ricin-resistant PC12 Cells Retained Normal Levels of Glycosyltransferase Activities—One possible defect leading to the

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**Fig. 1. Schematic representation of the experimental strategy.** N-linked oligosaccharides on cell surface glycoproteins that terminate in N-acetylglucosamine (the mannose core is not shown) can be labeled with [3H]galactose upon the addition of galacosyltransferase and UDP-[3H]galactose at 0 °C (exo-galactosylation). Upon reculture of the cells at 37 °C, glycoproteins may remain on the cell surface or may be transported to a variety of endocytic compartments. If galactosylated glycoproteins are transported to the Golgi apparatus, some or all of the galactose incorporated at the cell surface can be further glycosylated by endogenous glycosyltransferases. Galactosyltransferase can be measured in two ways as follows. A, acid precipitation from detergent lysates. Following reculture, detergent lysates are incubated with or without β-galactosidase. Terminal galactose residues are removed by the enzyme; additionally glycosylated galactose residues are resistant to digestion. Following digestion, the glycoproteins are acid-precipitated to separate them from free galactose. The precipitates are solubilized, and tritium counts recovered from digested and undigested samples are compared to quantitate glycosylation of galactose residues, or analyzed qualitatively by SDS-polyacrylamide gel electrophoresis and fluorography. B, analysis of isolated N-linked oligosaccharides. N-linked oligosaccharides are isolated from recultured cells by digestion of cell lysates with peptides:N-acetylgalactosaminidase, acid precipitation, and desalting of the oligosaccharides in the supernatant. The isolated oligosaccharides are digested exhaustively with β-galactosidase. Oligosaccharides are then separated from free galactose by gel filtration chromatography. ■, N-acetylglucosamine; •, [3H]galactose; ◯, additional terminal sugar residues (sialic acid, N-acetylglucosamine and/or fucose).

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**TABLE I**

*Lectin binding properties of PC12 A1 and A3 cells (ng of lectin bound per mg of cell protein)*

<table>
<thead>
<tr>
<th></th>
<th>PC12 wild type</th>
<th>PC12 A1</th>
<th>PC12 A3</th>
<th>A1/exogal</th>
<th>A3/exogal</th>
<th>BSI</th>
<th>BSI + GlcNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ricin binding</td>
<td>197</td>
<td>35</td>
<td>35</td>
<td>261</td>
<td>245</td>
<td>4.0</td>
<td>5.7</td>
</tr>
<tr>
<td>Ricin + Gal*</td>
<td>3.6</td>
<td>3.4</td>
<td>3.6</td>
<td>3.6</td>
<td>3.6</td>
<td>3.6</td>
<td>4.4</td>
</tr>
<tr>
<td>BSI</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>BSII</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>BSII + GlcNAc*</td>
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</tr>
</tbody>
</table>

*Competing sugars (galactose or N-acetylglucosamine) were included at a final concentration of 40 mg/ml.

* Cells were incubated with galactosyltransferase and 10 mM UDP-galactose for 60 min at 0 °C prior to washing and addition of radiolabeled lectin.

ND, not determined.
phenotype observed in ricin-resistant cells is a lack of galactosyltransferase activity. This was tested in several of the ricin-resistant PC12 clones. Most of the clones showed specific activities of galactosyltransferase approximately twice that of the wild type cells. None was found deficient in galactosyltransferase activity. The results of clones A1 and A2 are shown in Table II.

An essential requirement for the detection of glycoprotein recycling in galactosylation-deficient cells is that the cells be capable of adding additional sugar residues to experimentally galactosylated oligosaccharides when they do reach the Golgi apparatus. The most common addition to galactose residues on glycoproteins is sialic acid. Therefore, we tested the ricin-resistant clones for sialyltransferase activity. Of seven clones tested, the specific activities ranged from approximately half to approximately twice the wild type level of enzyme activity. Clones A1 and A3 exhibited specific activities slightly greater than wild type cells (Table II).

**Ricin-resistant PC12 Cells Exhibited Regulated Secretion—**

The final property required of the cells for this study is that they retained the regulated secretory pathway normally found in PC12 cells (35, 36). This was tested by monitoring the release of stored \(^3\)H]noradrenaline in response to membrane depolarization with potassium in combination with carbamylcholine chloride (25). Most of the clones tested released increased secretion of \(^3\)H]noradrenaline in response to stimulation similar in magnitude to that observed in wild type cells, confirming that the specializations required for regulated exocytosis are retained in the ricin-resistant cells (Table II).

**Galactose Was Incorporated into Glycoproteins and Glycolipids in Ricin-resistant PC12 Cells—**

Preliminary experiments using detergent lysates of labeled cells suggested that a fraction of the radiolabeled galactose was incorporated into glycolipid acceptors in ricin-resistant PC12 cells, and that the labeled glycolipids were acid-precipitable under the experimental conditions (see below). To characterize the nature of cell surface acceptors for galactose, PC12 A1 cells were exogalactosylated with radiolabeled galactose, washed thoroughly, and harvested. Cell pellets were homogenized and subjected to extraction in chloroform:methanol with sodium in combination with carbamylcholine chloride (25). Most of the clones tested exhibited increased secretion of \(^3\)H]noradrenaline in response to stimulation similar in magnitude to that observed in wild type cells, confirming that the specializations required for regulated exocytosis are retained in the ricin-resistant cells (Table II).

**Specific activities of ricin-resistant PC12 cells relative to wild type PC12 cells**

<table>
<thead>
<tr>
<th>Specific activities of ricin-resistant PC12 cells relative to wild type PC12 cells</th>
<th>PC12 A1</th>
<th>PC12 A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactosyltransferase</td>
<td>2.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Sialyltransferase</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Regulated secretion</td>
<td>0.83</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* Measured as cpm transferred to acceptor per mg of cell protein.

**TABLE II**

<table>
<thead>
<tr>
<th>Control</th>
<th>6 h 37°C</th>
<th>6 h 18°C Neuraminidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC12 A1</td>
<td>1.5 ± 0.5</td>
<td>16 ± 0.4</td>
</tr>
<tr>
<td>CHO clone 13</td>
<td>0.7 ± 0.5</td>
<td>1.6 ± 0.5</td>
</tr>
</tbody>
</table>

* Lysates from cells recultured for 6 h were digested with neuraminidase in addition to \(\beta\)-galactosidase (see "Materials and Methods" and "Results").

Of the radioactivity recovered in the aqueous phase, 70 ± 6% was acid-precipitable (approximately 11% of the total radioactivity). These results show that most of the galactose was incorporated into protein acceptors, while a significant fraction was incorporated into organic solvent-extractable, acid-insoluble acceptors, assumed to include glycolipids.

**Cell Surface Glycoproteins Acquired Resistance to \(\beta\)-Galactosidase upon Reculture at 37 °C—**

In order to begin to characterize the traffic of cell surface glycoproteins to the Golgi apparatus, PC12 A1 and A3 cells and CHO clone 13 cells were exogalactosylated with radiolabeled galactose as described under "Materials and Methods." Following the labeling, cells were either harvested and lysed in detergent immediately or were incubated in growth medium for up to 12 h at 37 °C prior to lysis. Radiolabeled galactose was analyzed either by digestion of intact glycoproteins (and glycolipids) in the lysates with \(\beta\)-galactosidase followed by acid precipitation to separate released galactose from \(\beta\)-galactosidase-resistant incorporated galactose, or by isolating N-linked oligosaccharides, digesting these with \(\beta\)-galactosidase, and separating the products by gel filtration chromatography.

For analysis of intact glycoproteins, portions of the lysates were digested exhaustively with \(\beta\)-galactosidase prior to acid precipitation and solubilization. Aliquots of the solubilized precipitates were counted (see Fig. 1). The results are summarized in Table III. Galactose incorporated into acid-precipitable material in cells harvested immediately after labeling was almost entirely sensitive to \(\beta\)-galactosidase digestion. This level of acid-precipitable radioactivity (<2% of the input) was defined as the background in the assay. Acid-precipitable \(\beta\)-galactosidase-resistant radioactive activity increased to approximately 1% of the total radioactivity in CHO clone 13 cells upon reculture for 6 h at 37 °C. However, in lysates from PC12 A1 cells recultured at 37 °C for 6 h approximately 16% of the incorporated galactose was resistant to \(\beta\)-galactosidase. PC12 A3 cells yielded essentially identical results (not shown), indicating that the findings are not peculiar to a single clone. After correcting for background, approximately 14% of the galactose incorporated into PC12 A1 surface glycoconjugates, and approximately 1% of the galactose incorporated into CHO clone 13 cells, acquired resistance to \(\beta\)-galactosidase after 6 h of reculture. Kinetic experiments indicated that acquisition of galactose was greatest in PC12 A3 cells relative to wild type cells, as shown in Table II.
of β-galactosidase resistance measured by acid precipitation was linear for at least 8 h in PC12 A1 cells, and reached 23% of the total acid-precipitable radioactivity by 12 h of chase (Fig. 4).

N-linked oligosaccharides were isolated from lysates by digestion with peptide:N-glycosidase F followed by acid precipitation and desalting of the supernatant. Approximately 85–90% of the incorporated radioactivity was recovered in the acid supernatant, and approximately 95% of this radioactive material eluted in the void volume and separated from free galactose, salts, and buffers on Sephadex G-25 gel filtration chromatography. These oligosaccharides were digested with β-galactosidase, and the resulting products analyzed by Sephadex G-25 chromatography. Radioactivity in β-galactosidase-treated oligosaccharides from cells harvested immediately after labeling eluted as a single peak corresponding to the elution position of free galactose (Fig. 3). In oligosaccharides from cells recultured for 2 h, some of the radioactivity eluted in a peak beginning at the void volume, and corresponding to the position of oligosaccharides isolated in the desalting step. With increasing times of reculture, a greater fraction of the radioactivity incorporated into oligosaccharides eluted in the faster peak after β-galactosidase treatment (Fig. 3). The increase in β-galactosidase resistance as measured by this method showed kinetics very similar to those seen by digestion of lysates followed by acid precipitation, but reached a lower level of 16% of the incorporated galactose after 12 h of chase (Fig. 4).

Resistance to β-Galactosidase Was Not Acquired upon Reculture at 19°C—The effect of reduced temperature on acquisition of β-galactosidase resistance was investigated to begin to define the site of terminal glycosylation of surface-labeled glycoproteins in PC12 cells. Vesicular traffic between certain organelles is sensitive to reduce temperature. While endocytosis continues at a reduced rate at temperatures below 20°C, delivery of endocytic contents to lysosomes is inhibited (37, 38). More importantly, the transport of ricin toxin from endosomes to the Golgi apparatus (39, 40) and the transport of cell surface glycoproteins to the site of sialyltransferase activity (1, 3, 4, 6) are also efficiently blocked below 20°C. When ricin-resistant PC12 cells were incubated at 19°C for 6 h following exogalactosylation, the fraction of galactose resistant to β-galactosidase, either in total acid-precipitable material (Table III), or isolated N-linked oligosaccharides (not shown) was only slightly greater than the fraction found in cells harvested immediately following labeling. These experiments show that galactose residues were not modified on the cell surface or in endosomes in ricin-resistant PC12 cells, and are consistent with a requirement for transport to the Golgi apparatus. Finally, PC12 A1 cells recultured at 37°C in medium containing 10 μg/ml cycloheximide exhibited the same level of β-galactosidase-resistant radiolabeled isolated oligosaccharides and in acid-precipitable products from lysates as cells recultured without cycloheximide (not shown). These results preclude the possibility that radiolabeled galactose was incorporated biosynthetically from a pool liberated from glycoproteins that were degraded in lysosomes during reculture.

The experiments described above demonstrate that the galactose incorporated into glycoconjugates at the cell surface in ricin-resistant PC12 cells becomes resistant to β-galactosidase upon reculture as a result of additional terminal glycosylation of the galactose residues, and that the glycosylation occurs in an endocytic compartment not accessible at 19°C. This compartment is most likely the Golgi apparatus (2, 41)

PC12 A1 Cells Added Several Different Sugar Residues to Galactose Incorporated into N-linked Oligosaccharides—Published reports (1, 3, 4, 5, 6) on transport of glycoproteins from the cell surface to the Golgi apparatus have demonstrated the addition of sialic acid residues. If sialic acid is the only sugar residue incorporated into exogalactosylated glycoproteins, addition of neuraminidase to the β-galactosidase digestion should result in the removal of all of the radiolabeled galactose. When this experiment was performed by acid precipitation of digested lysate samples from PC12 A1 or A3 cells,
using Vibrio cholerae and Clostridium perfringens neuraminidases, approximately one third of the β-galactosidase resistant galactose was removed (Table III). Thus, in contrast to earlier biochemical studies of endocytic Golgi traffic, PC12 cells appeared to add sugar residues in addition to sialic acid to galactosylated glycoproteins.

In order to determine the nature of the modifications accounting for resistance to β-galactosidase, radiolabeled N-linked oligosaccharides were isolated from PC12 A1 cells recultured for 6 h after exogalactosylation. These oligosaccharides were digested with β-galactosidase with or without a variety of additional glycosidases, and with or without prior mild acid hydrolysis to remove completely sialic acid residues. The products were separated by gel filtration chromatography, and the radioactivity in the two peaks was counted as described above. The percentage of counts eluting in the first peak (β-galactosidase resistant) in each sample was normalized to the percentage in the sample digested with β-galactosidase alone. The results are summarized in Table IV. For simplicity, it will be understood in the following description that all samples were digested with β-galactosidase.

Removal of sialic acid residues by neuraminidase digestion or acid hydrolysis alone converted approximately one-quarter of the β-galactosidase resistant galactose in isolated oligosaccharides to β-galactosidase-sensitive. Addition of β-N-acetylglucosaminidase alone exposed approximately one-quarter of the protected galactose. Removal of sialic acid residues and N-acetylglucosamine residues simultaneously by combining these treatments removed approximately half of the protected galactose residues. The additive effects of these two treatments suggest that upon reculturing the cells sialic acid was added to some of the galactose residues incorporated at the cell surface, and N-acetylglucosamine was added to others. Addition of N-acetylglucosamine to galactose to form N-acetyllactosamine disaccharide units can occur both on glycoproteins and glycolipids, and can exist as tandem repeating disaccharide units (polylactosamine) terminating either in sialic acid or in α-linked galactose (42). Such polylactosamine repeats have been described in PC12 cells (43). The generation of lactosamine structures on these oligosaccharides was confirmed by treatment with endo-β-galactosidase, which was more effective than β-N-acetylglucosaminidase at unblocking and removing protected galactose. (Assuming that the lactosamine units in these cells terminated in N-acetyllactosamine, the product of endo-β-galactosidase digestion would be a disaccharide, which would elute in the second peak on gel filtration chromatography.)

A well characterized modification of N-acetyllactosamine and galactose residues is the addition of fucose residues. Addition of fucose or other sugar residues renders both N-acetyllactosamine and lactosamine relatively resistant to their cognate glycosidases (44, 45). To assess the role of fucose addition in protecting galactose residues on the isolated oligosaccharides, α-fucosidase was included in some digestions. Including α-fucosidase substantially decreased the fraction of protected galactose in the presence or absence of β-N-acetylglucosaminidase or endo-β-galactosidase (Table IV). Since the α-fucosidase used in this study cleaves both α1,2 and α1,3-linked fucose residues (46), fucose residues linked α1,3 to terminal N-acetyllactosamine (47-49) or α1,2 to galactose (47) would have been removed. The presence of significant β-N-acetyllactosaminidase activity in the α-fucosidase used in this work raises the possibility that the effect of α-fucosidase in the absence of added β-N-acetyllactosaminidase may have been at least partially due to this contaminating activity. Thus, we conclude that fucose residues were added to N-acetyllactosamine residues and/or to galactose residues in recultured PC12 A1 cells.

Combining several of the treatments described above in order to remove sialic acid, N-acetyllactosamine, and fucose residues resulted in the conversion of approximately 87% of the resistant galactose to β-galactosidase sensitive. The small fraction of β-galactosidase-resistant galactose remaining under these conditions could be blocked by other covalent modifications, or could be due to decreased efficiency in some of the enzymes in an increasingly dilute and complex solution. In summary, it was found that the presence of sialic acid residues alone accounted for approximately one quarter of the observed β-galactosidase resistance in isolated oligosaccharides from recultured PC12 A1 cells. Terminal N-acetyllactosamine accounted for an additional one-quarter, and fucose addition to N-acetyllactosamine and/or galactose residues accounted for at least 40% of the observed resistance. All of these modifications to experimentally added galactose residues upon reculture of the cells are consistent with transport of glycoproteins from the cell surface to the Golgi apparatus. PC12 A1 Cells Transported Many More Proteins to the Site of Sialytransferase Than Did CHO clone 13 Cells—It has been clearly demonstrated that transport to the Golgi apparatus from the cell surface is highly selective in nonsecretory cell types (3, 5, 6). This aspect of glycoprotein transport was addressed by comparing the proteins that were terminally glycosylated after exogalactosylation in PC12 A1 cells and CHO clone 13 cells. Samples from the quantitation experiments presented in Table III were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Based on the data obtained by counting radioactivity in aliquots of the solubilized acid precipitates, equal counts of radioactivity were loaded on the gels where possible. Samples that contained too few counts were analyzed in entirety. An important consequence of loading equal counts of radioactivity is this:

<p>| Glycosidase Analysis of Asparagine-Linked Oligosaccharides Isolated from Recultured PC12 A1 Cells: Fraction of [%H]Galactose Residues Incorporates into Asparagine-Linked Oligosaccharides That Were Resistant to Digestion with β-Galactosidase Alone or in Combination with Other Glycosidases, Relative to the Fraction Resistant to β-Galactosidase Alone |
|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>PC12 A1 cells were exogalactosylated with UTP- [%H]galactose and recultured for 6 h at 37 °C. Asparagine-linked oligosaccharides were isolated, aliquots dissolved in 50 mM MES, pH 6.0, and digested exhaustively with the indicated glycosidases as described under &quot;Materials and Methods.&quot; For acid hydrolysis, isolated oligosaccharides were dissolved in 10 μL of 2 M acetic acid, incubated 60 min in a boiling water bath, neutralized with 2 μL of 10 N NaOH, and diluted 10-fold in 90 mM MES, pH 6.0, prior to addition of glycosidases. Following digestion, samples were boiled for 3 min to inactivate the enzymes and analyzed by gel filtration chromatography on Sephadex G-25. Counts eluting in a peak immediately following the void volume were counted as resistant to digestion, and the large peak of counts eluting in later fractions counted as sensitive to digestion (see Fig. 3). The percentages of counts eluting in the first peak were normalized to the percentage obtained when oligosaccharides were digested with β-galactosidase alone.</th>
<th>+ β-Galactosidase</th>
<th>+ β-Galactosidase and α-Fucosidase</th>
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<tbody>
<tr>
<td>No additional treatment</td>
<td>1.00</td>
<td>0.66</td>
</tr>
<tr>
<td>Acid hydrolysis</td>
<td>0.77</td>
<td>ND*</td>
</tr>
<tr>
<td>Neuraminidases</td>
<td>0.77</td>
<td>ND*</td>
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<tr>
<td>β-N-Acetylglucosaminidase</td>
<td>0.76</td>
<td>0.40</td>
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<tr>
<td>Acid hydrolysis, and then β-N-acetylglucosaminidase</td>
<td>0.51</td>
<td>ND*</td>
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<tr>
<td>β-N-Acetylglucosaminidase and neuraminidases</td>
<td>0.46</td>
<td>0.13</td>
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<tr>
<td>Endo-β-galactosidase</td>
<td>0.59</td>
<td>0.30</td>
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* ND, not determined.
Where equal counts of β-galactosidase-resistant undigested proteins were analyzed (PC12 A1 cells), a band representing a glycoprotein that is additionally glycosylated with an efficiency equal to the average for the total (14% in 6 h); would appear with equal intensity in the digested and undigested samples. Bands of greater intensity in the β-galactosidase-digested samples relative to the undigested samples represent glycoproteins glycosylated with greater efficiency than the average for the total sample, and fainter bands correspond to glycoproteins glycosylated with efficiencies less than the average.

The results of this analysis are shown in Fig. 5, in which the relative protein loads in each lane are indicated. A similar number of bands in PC12 A1 cells, which yielded insufficient precipitable radioactivity was incorporated into material that migrated ahead of the dye front (Fig. 5, 6-h chase, + β-galactosidase). This material was not seen in samples containing equal total radioactivity (one-seventh as much precipitated lysate) that were not digested, nor was it detected in any samples from CHO clone 13 cells.

In CHO clone 13 cells, 85 times more β-galactosidase-digested protein than undigested protein was required to detect a few faint bands of low mobility (in the experiment shown, only 10.5 times as much digested protein was loaded on the gel). The bands were much more numerous and prominent in the PC12 A1 samples under the same conditions (Fig. 5, 6-h chase, + β-galactosidase). Most of the bands that were prominent in the undigested samples from PC12 A1 cells were faint or undetected in the digested sample, indicating that these proteins were not glycosylated efficiently. Other bands that were not prominent in the undigested samples were considerably enhanced in the digested sample (arrows). Analysis of PC12 A3 cells yielded similar results (not shown). Thus, the transport of cell surface glycoproteins to the site of glycosyltransferase activities in PC12 cells was highly selective, efficiently including few if any of the major labeled glycoproteins. In contrast to CHO clone 13 cells, many more proteins that were not among the major labeled proteins acquired significant resistance to β-galactosidase in PC12 A1 cells.

In PC12 A1 cells that had been recultured for 6 h, acid precipitable radioactivity was incorporated into material that migrated ahead of the dye front (Fig. 5, 6-h chase, + β-galactosidase). This material was not seen in samples containing equal total radioactivity (one-seventh as much precipitated lysate) that were not digested, nor was it detected in any samples from CHO clone 13 cells. This radioactivity may represent glycolipids that are efficiently acid-precipitated, and resistant to β-galactosidase digestion under the conditions employed in this experiment. If this radiolabeled material proves to be glycolipid, the ricin-resistant PC12 cells can be used to monitor transport of endogenous glycolipid as well as glycoprotein to the Golgi apparatus.

**DISCUSSION**

The ricin-resistant PC12 cells described in this report constitute the first system for measuring glycoprotein traffic from the cell surface to the Golgi apparatus biochemically in regulated secretory cells. As described previously for the mannose 6-phosphate receptors in nonsecretory cell lines (3, 7), some of the radiolabeled galactose incorporated into a subset of PC12 cell surface glycoproteins acquired additional terminal sugar residues upon reculturing the cells. Terminal glycosylation was not observed when ricin-resistant PC12 cells were recultured at 19 °C, a condition that allows endocytic transport from the cell surface to endosomes, but prevents transfer to lysosomes (37, 38) and the Golgi apparatus (4, 6, 39, 40). These results strongly suggest that the acquisition of resistance to β-galactosidase we have measured reflects transport of cell surface glycoproteins to the Golgi apparatus in endocrine cells.

The nature of the defect in ricin-resistant PC12 cells has not yet been defined. The PC12 mutants tested all had normal or slightly elevated levels of galactosyltransferase activity. Since the nonspecific galactose acceptor ovalbumin was used in this assay, a defect in β-N-acetylgalactosaminide β1,4-galactosyltransferase, the enzyme normally active in glycosylation of N-acetylgalactosamine residues in N-linked oligosaccharides, cannot be definitively ruled out (50, 51). However, the resistance of these cells to ricin toxin strongly suggests that they incorporated few galactose residues into any acceptors by any mechanism. At least two cell lines, CHO clone 13 (34) and MDCK (23), exhibiting the same glycosylation phenotype have been shown to be deficient in the transport of UDP-galactose from the cytoplasm to the lumen of the Golgi apparatus, and several other mutant cell lines display similar glycosylation phenotypes (52). A different genotype with the
same phenotype is a defect in the UDP-galactose, UDP-
agalactosamine 4-epimerase (53). While the nature of the de-
defect in PC12 mutants awaits definitive identification, the
phenotype is clearly appropriate for the studies reported here.

In contrast to earlier results in none secretory cell types, galactose incorporated into N-linked oligosaccharides ac-
quired N-acetylgalactosamine and fucose residues in addition
to sialic acid residues in ricin-resistant PC12 cells. In many
cell types, sialyltransferase activity is localized primarily to the
trans Golgi cisterna and trans Golgi network (TGN), although several cell types have sialyltransferase in other
Golgi cisternae (2). Therefore, addition of sialic acid residues alone to galactose incorporated at the cell surface has been
interpreted as evidence for endocytic transport to the late
Golgi. The site of synthesis of polylactosamine structures has not been as rigorously localized, but requires the simultaneous
presence of galactoside:1,3-N-acetylglucosaminyltransferase
and of 1-acetylglucosaminide:1,4-galactosyltransferase. The
latter is generally localized to the trans Golgi cisterna(e) (2,
42). Similarly, addition of fucose-linked α1,6 core 1-acetyl-
galactosamine residues occurs in medial Golgi compartments
(42), but addition of fucose to galactose and to terminal 1-acetyl-
galactosamine residues in polylactosamine repeats cannot
precede the addition of galactose in the trans Golgi cisterna(e).
Thus, while the Golgi subcompartment that receives endocy-
totic traffic in PC12 cells cannot yet be identified with certainty,
it is likely a late Golgi compartment, presumably the trans
cisterna(e) and/or the TGN. Whether this represents a sig-
nificant difference in membrane targeting between non-secret-
ory cell types and PC12 (and possibly other secretory cells),
or simply reflects differences in glycosyltransferase distribu-
tion in PC12 cells remains to be determined.

Several assumptions are inherent in the design and inter-
pretation of these experiments. It is assumed that the range
of structures of complex N-linked oligosaccharides are similar
on all of the glycoproteins produced by a given cell type, that
the exogalactosylation reaction labels all cell surface oligosac-
charides with equal efficiency, and that all galactosylated
oligosaccharides are equally good substrates for additional
glycosylation, if they are transported to the Golgi apparatus.
While it is likely that none of these assumptions is strictly
true (42), they probably represent a reasonable approximation
for many glycoproteins. However, Margolis et al (43), using
endo-β-galactosidase to expose acceptor sites for exogalaco-
sylation, have presented data suggesting that many, but not
all glycoproteins in PC12 cells carry polylactosamine-contain-
ing oligosaccharides. Finally, it is not known whether a single
round of transport to the Golgi apparatus is sufficient to
glycosylate fully all of the labeled galactose incorporated into
oligosaccharides at the cell surface, or whether all of the
galactose residues are substrates for additional glycosylation.
If either is not the case, the percentage of galactose protected
from β-galactosidase would be an underestimate of the per-
centage of galactosylated molecules that have returned to the
Golgi apparatus.

Transport of cell surface glycoproteins to the Golgi appa-
ratus appeared to be quantitatively much greater in ricin-
resistant PC12 cells than in the nonendocrine CHO clone 13
cells and involved many more glycoproteins. While a signif-
icant fraction of the mannose 6-phosphate receptors at the
surface are transported to the TGN in CHO and other
none secretory cells (3, 4), no significant transport of total cell
surface glycoproteins to the Golgi apparatus has been detected
in several cell types using several different methods (3, 5, 6,
18). In ricin resistant PC12 cells at least 18% of the cell
surface N-linked oligosaccharides were transported to the
Golgi apparatus after 12 h. The dramatic difference between
PC12 cells and other cell types in the magnitude of glycosyl-
ation of cell surface galactose residues may merely reflect a
more efficient action of a greater variety of glycosyltrans-
ferases on the same number of substrates. If this is so, the
measurements made in other cell types may have significantly
underestimated the actual efficiency of this pathway. An
alternative explanation is that the presence of a regulated
secretory pathway in PC12 cells may amplify the endocytic
Golgi pathway due to the recycling of secretory granule mem-
brane components. The latter hypothesis is consistent with the
demonstration that secretory vesicle membrane is turned
over more slowly than the vesicle contents are secreted (11),
implying that the vesicle proteins are reutilized. The current
data cannot definitively distinguish between these alter-
atives.

A fraction of the galactose incorporated into acceptors other
than N-linked oligosaccharides in ricin-resistant PC12 cells
exhibited properties suggesting that the acceptors were gly-
colipids. This included up to 7% of the acid-precipitable β-
galactosidase-resistant galactose detected after 12 h of recul-
ture, a fraction of which migrated ahead of the dye front on
SDS-polyacrylamide gel electrophoresis. (It should be noted
that the conditions employed in this study are not optimal
for glycosidase treatment of glycolipids.) The little that is
known about glycolipid traffic is consistent with its recycling
from the cell surface to the Golgi apparatus. It has recently
been shown that Shiga toxin, which binds to cell surface
glycolipids, is internalized via clathrin-coated pits and tar-
ged in part to the Golgi apparatus (54). Cholera toxin, which
binds to the ganglioside GM2, has also been traced to the Golgi
region (55). Glycolipid and sphingomyelin analogs incorpo-
rated into the cell surface can be internalized and recycled
along the same pathway as transferrin (56, 57), and exogenous
or endogenous glycolipids are sialylated at least in part by
pathways independent of lysosomal turnover (58-60). If the
galactose that is not in N-linked oligosaccharides in PC12
cells is in glycolipids, the ricin-resistant PC12 cells would
represent a powerful system for the study of glycolipid traffic.

Part of the impetus for the current work came from a
comparison of morphological studies of traffic from the
cell surface to the Golgi apparatus in secretory and none secretory
cell types. Due to the nature of the markers used in these
studies, it was not possible to identify the specific elements
of the pathway. Using the ricin-resistant PC12 cells described
in this report, a quantitative analysis of endocytic transport
to the Golgi apparatus for specific membrane markers is now
feasible. Such studies may elucidate the relative contributions
of different organelles, and the importance of the regulated
secretory pathway, in generating this membrane transport
pathway. These studies will complement both the morpholog-
ical studies in regulated secretory cells and the quantitative
biochemical data previously obtained in none secretory cell
types.

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Endocytic membrane traffic to the Golgi apparatus in a regulated secretory cell line.

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