

Membrane Traffic in Animal Cells: Cellular Glycoproteins Return to the Site of Golgi Mannosidase I

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Abstract. The recycling of cellular glycoproteins to the site of Golgi mannosidase I, an enzyme of asparagine-linked oligosaccharide synthesis, was studied in K562 human erythroleukemia cells. Cells were metabolically labeled in the presence of deoxymannojirimycin, a reversible inhibitor of Golgi mannosidase I. This generates glycoproteins with immature oligosaccharides in their normal locations. Transport to the mannosidase I compartment was then assessed by testing for the conversion of oligosaccharides into mature forms during reculture without deoxymannojirimycin.

Transferrin receptor (TfR) was acted on by mannosidase I during reculture, suggesting that it returned to

the region of the Golgi complex where this enzyme resides. The slow rate of this transport ($t_{1/2} > 6$ h) implies that it is probably different than TfR movement during transferrin internalization ($t_{1/2} = 10$ – 20 min) and TfR transport to the sialyltransferase compartment in the Golgi complex ($t_{1/2} = 2$ – 3 h) (Snider, M. D., and O. C. Rogers, 1985, *J. Cell Biol.*, 100:826–834). The total cell glycoprotein pool was also transported to the mannosidase I compartment with a half-time of 4 h. Because this transport is 5–10 times faster than the rate of *de novo* glycoprotein synthesis in these cells, it is likely that most of the glycoprotein traffic through the Golgi complex is composed of recycling molecules.

THE plasma membrane of animal cells is connected to intracellular membrane-bound organelles by complex pathways of membrane traffic. During endocytosis constituents move from the plasma membrane to endosomes, phagosomes, lysosomes, and the Golgi complex (19, 24, 52). Similarly, during secretion, membrane and soluble proteins move from the endoplasmic reticulum (ER)¹ through the Golgi complex to the cell surface (19).

We have been using a new approach to examine the movement of surface proteins through intracellular organelles. First, a covalent modification is made to the proteins so that they are substrates for an enzyme of known intracellular location. Transport into the enzyme-containing compartment can then be studied by monitoring the covalent structure of the proteins.

This approach is being used to examine the entry of surface glycoproteins into the Golgi complex, using enzymes of glycoprotein oligosaccharide synthesis found in this organelle. Glycoproteins with oligosaccharides that are substrates for Golgi enzymes are generated on the cell surface. The structures of the glycoprotein oligosaccharides are then monitored to test entry into enzyme-containing compartments. In

a previous study, we used this approach to show that surface transferrin receptor (TfR) is transported from the cell surface to a sialyltransferase-containing compartment, most likely in the *trans* region of the Golgi complex (51).

In this study, we tested glycoprotein entry into compartments that contain the Golgi enzyme mannosidase I, an enzyme of asparagine-linked oligosaccharide synthesis. All asparagine-linked oligosaccharides are derived from the precursor $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, which is added to peptide chains in the rough ER. This precursor is then converted to mature structures as the glycoprotein is transported through the ER and Golgi region (summarized in Fig. 1). First, ER glucosidases act to form the high mannose species $\text{Man}_9\text{GlcNAc}_2$. Then four α 1-2-linked mannose (Man) residues are removed to generate $\text{Man}_5\text{GlcNAc}_2$, in a process termed Man trimming. Several mannosidases in the ER and Golgi region carry out this trimming (4, 20, 55, 57), with the bulk of the activity due to Golgi mannosidase I. The $\text{Man}_5\text{GlcNAc}_2$ intermediate can then be further modified by the addition of peripheral *N*-acetylglucosamine (GlcNAc), galactose and sialic acid residues to generate mature complex and hybrid asparagine-linked oligosaccharides (for review see references 35 and 50).

To test the entry of glycoproteins into the compartment where Man trimming occurs, we have used deoxymannojirimycin (dMM), a mannose analogue that reversibly in-

1. *Abbreviations used in this paper:* dMM, deoxymannojirimycin; ER, endoplasmic reticulum; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; PMSF, phenylmethylsulfonyl fluoride; TfR, transferrin receptor; TfR^{dMM}, TfR synthesized in the presence of dMM.

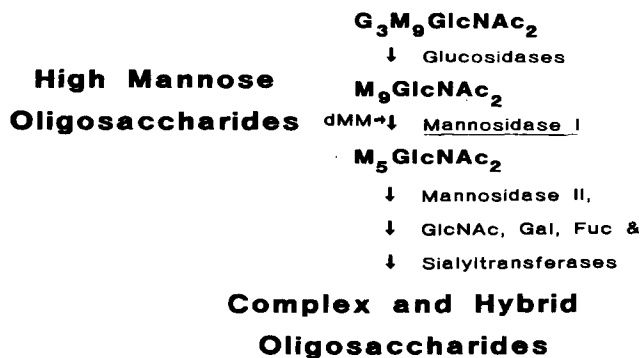


Figure 1. Processing of asparagine-linked oligosaccharides. After the precursor oligosaccharide is transferred to peptides in the ER, glucosidases remove the three glucose (Glc) residues in this organelle. Then four Man residues are removed, primarily by dMM-sensitive Golgi mannosidase I. Finally, terminal processing enzymes in the Golgi complex convert high mannose oligosaccharides to complex and hybrid structures. G, glucose; M, mannose; GlcNAc, N-acetylglucosamine.

hibits Golgi mannosidase I (5, 17, 21). Cells are metabolically labeled and then chased, all in the presence of dMM to generate labeled glycoproteins in their normal locations with immature oligosaccharides. The dMM is then removed and entry of the glycoproteins into mannosidase I-containing compartments is tested by determining whether the immature oligosaccharides have been converted to mature forms.

The transport of both TfR and the total glycoprotein pool in K562 human erythroleukemia cells were examined in this study. TfR is a membrane glycoprotein that functions in the uptake of iron from the plasma iron carrier, transferrin. Iron-transferrin binds to TfR on the cell surface and the complex is internalized via coated vesicles. The iron then dissociates, most likely in the acidic endosomal compartment, and the complex returns to the cell surface where apotransferrin is released (11, 33). TfR is a ubiquitous cell surface glycoprotein composed of two identical disulfide-linked subunits of $M_r \sim 90,000$ each (40, 48, 53).

We show that TfR returns slowly to the mannosidase I compartment. Moreover, after entering this compartment, receptors appear to follow the normal exocytic pathway through the Golgi complex, where they are exposed to later-acting enzymes of oligosaccharide synthesis. We also find that a substantial portion of the total cell glycoprotein pool returns to the mannosidase I compartment. Because this transport is significantly faster than the rate of *de novo* synthesis, most of the glycoproteins transported through the mannosidase I compartment are probably recycling molecules.

Materials and Methods

Cell Culture

K562 human erythroleukemia cells were grown in suspension in α -minimal essential medium supplemented with 10% fetal bovine serum as previously described (51). HeLa S3 cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in spinner culture in Joklik-modified minimal essential medium and 10% fetal bovine serum.

Labeling of Cells

Cells were metabolically labeled with [35 S]methionine in Met-free mini-

mal essential medium supplemented with non-essential amino acids and 5% dialyzed fetal bovine serum. After centrifugation from the culture medium, cells were washed twice with labeling medium (1 ml/5 $\times 10^6$ cells). Cells (1.33 $\times 10^7$ /ml) were then incubated with 160 μ Ci/ml [35 S]Met (1,200 Ci/mmol, Amersham Corp., Arlington Heights, IL) for 30 min. Labeling was terminated by adding growth medium (1 ml/5 $\times 10^6$ cells). The cells were then centrifuged, and chased in fresh growth medium for 2 h.

Cells were labeled with [3 H]Man in minimal essential medium with the glucose concentration reduced to 0.1 mg/ml, supplemented with non-essential amino acids and 5% dialyzed fetal bovine serum. Cells were washed twice with this medium and then incubated with 0.4 mCi/ml [$2-^3$ H]Man (14 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) at a cell density of 1.33 $\times 10^7$ /ml for 45 min. Cells were then centrifuged and chased for 3 h in fresh growth medium (5 $\times 10^6$ cells/ml).

Deoxymannojirimycin (dMM) (the generous gift of Dr. Gunter Legler, University of Köln), was stored as a 30-mM aqueous solution at -20°C . Cells were pulsed and chased in media containing 0.5 mM dMM. At the end of the chase period, the cells were centrifuged and recultured at 37°C in dMM-free growth medium. Swainsonine (the gift of Dr. Oscar Touster, Vanderbilt University) was added to culture medium at 1 μ g/ml. Tunicamycin (Calbiochem-Behring Corp., La Jolla, CA) was dissolved in dimethylsulfoxide and added to 20 μ g/ml. This concentration inhibited the glycosylation of TfR by >90% (not shown). Cycloheximide (Sigma Chemical Co., St. Louis, MO) was added at 10 μ g/ml, a concentration that inhibited protein synthesis by >95%. The above incubations were all performed at 37°C in a 5% CO_2 atmosphere.

Immunoprecipitation of TfR

Cells were lysed and TfR immunoprecipitated using B3/25 anti-human transferrin receptor monoclonal antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN), goat anti-mouse IgG and fixed *Staphylococcus aureus* cells (the gift of Dr. Douglas Fambrough, The Johns Hopkins University), all as previously described (51). For the analysis of [35 S-Met]TfR, immunoprecipitates from 2.5 $\times 10^6$ cells were dissociated by heating for 5 min at 95°C in 50 μ l of 0.1 M sodium citrate, pH 5.5, 1% SDS, 5% 2-mercaptoethanol, 2 mM NaN_3 . After centrifugation at 13,000 g for 5 min, duplicate 25- μ l samples were taken from each tube and mixed with an equal volume of water. Endo H (30 ng) from *Streptomyces griseus* (the gift of Dr. P. W. Robbins, Massachusetts Institute of Technology) was added to one of the duplicate samples and all the tubes incubated at 37°C for 16 h. SDS gel sample buffer (25 μ l of 3x buffer) was then added, the samples heated to 95°C for 5 min and analyzed on 7.5% SDS polyacrylamide gels (36). Gels were fixed in 10% acetic acid, 25% isopropanol, soaked in Enlightening (New England Nuclear, Boston, MA), dried, and visualized by fluorography. Comparison of samples incubated at 37°C without endo H and unincubated samples showed that the incubation itself had no effect on TfR forms (not shown).

Analysis of Glycopeptides

[3 H]Man-labeled samples (5 $\times 10^6$ cells) were lysed and TfR immunoprecipitated. Immune complexes were suspended in 100 μ l of 0.1 M Tris HCl, pH 8.0, 2 mM dithiothreitol, 1 mM NaN_3 , and incubated with Pronase (20 μ l, 20 mg/ml) at 50°C for 36 h. Additional pronase was added after 12 and 24 h of incubation. Samples were then heated at 95°C for 5 min and equilibrated with 0.1 M Na citrate, pH 5.5, 1 mM NaN_3 by centrifugation through columns of Sephadex G-10 swollen in the same buffer. Samples were incubated with endo H (30 ng) for 16 h at 37°C and then heated for 5 min at 95°C .

The resulting oligosaccharides and glycopeptides were analyzed by gel filtration chromatography on columns of Bio-Gel P-4 (-400 mesh, 1×120 cm) using bovine serum albumin and [3 H]Man as markers for the excluded and included volumes, respectively. Peaks were identified by comparison with authentic standards and by calculation of the relative elution coefficient, K_D , all as previously described (31).

To analyze cell lysates and immunosupernatants, 0.2 mg ovalbumin was added, and the proteins were precipitated with trichloroacetic acid (10% final). Samples were centrifuged for 5 min at 13,000 g, and the pellets washed twice with 0.5 ml ice cold acetone. The pellets were dried, digested with pronase, with endo H, and then analyzed as described above.

Determination of Cell Surface TfR

Labeled cells were chilled and washed twice with ice cold Dulbecco's phosphate-buffered saline (PBS). Aliquots of 10^6 cells in PBS were then

incubated with 2.5 μg of trypsin (TRTPCK, Worthington Biochemical Corp., Freehold, NJ) for 60 min on ice. PBS (0.5 ml) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) was then added, the samples were centrifuged, and the cell pellet was washed once with PMSF-containing PBS. Cells were then lysed and TfR immunoprecipitated in buffers containing 1 mM PMSF. Samples were analyzed by SDS gel electrophoresis and the amount of TfR quantitated by scanning densitometry. The recovery of TfR was compared to samples incubated without protease. To ensure that the observed proteolysis had occurred during trypsin treatment of intact cells and not in cell lysates during TfR immunoprecipitation, controls were incubated on ice for 60 min without protease. Trypsin was then added immediately after the addition of the PMSF-containing PBS, and TfR was immunoprecipitated and analyzed. No proteolysis of TfR was observed under these conditions (not shown).

Binding and Internalization of Transferrin

Human transferrin was saturated with iron (10) and then iodinated to a specific activity of 6×10^6 cpm/ μg using Na^{125}I and Iodo-Gen (Pierce Chemical Co., Rockford, IL). Binding was performed in PBS containing 1% fetal bovine serum and 1 mg/ml glucose (binding medium). Control cells, and cells that had been cultured for 24 h with 0.5 mM dMM were centrifuged, washed twice with ice cold binding medium, and then incubated on ice with 3 $\mu\text{g}/\text{ml}$ ^{125}I -transferrin at a cell density of $5 \times 10^6/\text{ml}$. Cells were then washed twice with ice cold binding medium, resuspended in warm binding medium and incubated at 37°C. Binding was measured essentially as described by Klausner et al. (34). Total cell-associated ^{125}I -transferrin was measured by centrifuging samples (8×10^5 cells) through dibutylphthalate/mineral oil (9:1, vol/vol), and determining the radioactivity in the cell pellet with a gamma counter. Released transferrin was measured by mixing supernatants with an equal volume of 1% phosphotungstic acid, 0.5 M HCl, centrifuging, and counting the pellet. SDS gel electrophoresis showed that all the acid-insoluble material released into the medium migrated with authentic transferrin (not shown). Internal transferrin was measured by treating cell aliquots briefly with acid to dissociate surface ligand, and then neutralizing and centrifuging through oil (34). Surface transferrin was calculated as the difference between total and internal cell-associated transferrin. Values were corrected for nonspecific binding, measured in the presence of a 100-fold excess of unlabeled transferrin.

Results

dMM Reversibly Inhibits Mannosidase I

While the inhibition of Golgi mannosidase I by dMM is well documented (5, 17, 21), the reversibility of this inhibition has not been examined. To establish that the inhibition is reversible, we examined the effect of dMM on the synthesis of TfR oligosaccharides in K562 cells. Cells were labeled with [^{35}S]Met and TfR was immunoprecipitated. Portions of each sample were incubated with endo H, which cleaves high mannose but not complex oligosaccharides. Endo H-treated and untreated samples were then analyzed on SDS gels. Fig. 2 A shows TfR from cells pulsed for 30 min. A single polypeptide of apparent M_r 85,000 was seen. This polypeptide had endo H-sensitive oligosaccharides, since treatment caused a substantial increase in mobility on the gel. TfR from cells pulse-labeled for 30 min and chased for 2 h in unlabeled medium is shown in Fig. 2 B. This TfR migrated with apparent M_r \sim 90,000 as a broad band. At least one of the asparagine-linked oligosaccharides had been converted to an endo H-resistant form, since the increase in mobility on endo H treatment was less than in sample A. However, this mature TfR retains at least one endo H-sensitive oligosaccharide. These results are identical to previously reported findings with TfR in other human cell lines (40, 47).

Fig. 2 C shows TfR from cells pulsed and chased in the presence of dMM. The synthesis of endo H-resistant oligosaccharides was blocked, since TfR mobility and sensitiv-

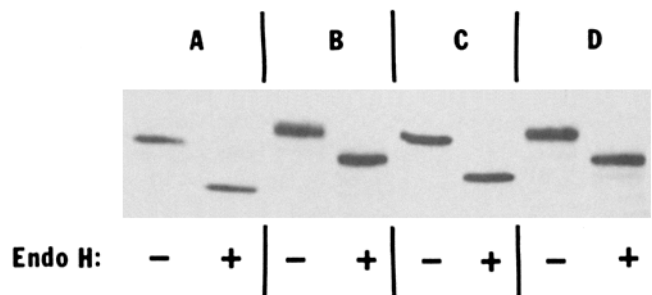


Figure 2. dMM inhibits mannosidase I reversibly. Cells were labeled with [^{35}S]Met and TfR was immunoprecipitated as described in Materials and Methods. A portion of each sample was treated with endo H and analyzed on SDS gels. A fluorograph of the dried gel is shown. (A) Cells were pulsed with [^{35}S]Met for 30 min. (B) Cells were pulsed with [^{35}S]Met for 30 min and then chased for 2 h. (C) Cells were pulsed with [^{35}S]Met for 30 min and chased for 2 h, all in the presence of 0.5 mM dMM. (D) Cells were incubated with 0.5 mM dMM for 2.5 h, washed, and then pulsed with [^{35}S]Met and chased in dMM-free medium.

ity to endo H were similar to that seen in pulse-labeled cells (Fig. 2 A). This TfR migrated more slowly than pulse-labeled receptor. The difference could be due to the presence of phosphate (47) and fatty acid residues (40), which are added to TfR during maturation. TfR synthesized in the presence of dMM will be referred to as TfR^{dMM}.

Fig. 2 D shows that the inhibition of mannosidase I by dMM is reversible. Cells were treated with dMM for 2.5 h, the length of time required for the pulse-chase labeling. The cells were then washed, pulsed with [^{35}S]Met, and chased, all in dMM-free medium. The major TfR species was the mature form found in control cells. TfR^{dMM} made up only a small fraction of the labeled receptor, indicating that mannosidase I is reversibly inhibited by dMM under the conditions used. In similar experiments, we observed that the inhibition of asparagine-linked oligosaccharide assembly is also reversible after 24 h of dMM treatment (not shown).

TfR^{dMM} Returns to the Site of Mannosidase I

To test the reentry of TfR into mannosidase I-containing compartments, K562 cells were pulsed with [^3H]Man and chased for 3 h, all in the presence of dMM. The inhibitor was then removed and the cells recultured in growth medium. Glycopeptides were prepared from ^3H -TfR and analyzed by gel filtration chromatography on Bio-Gel P-4 (Fig. 3).

TfR in control cells contained several types of asparagine-linked oligosaccharides (Fig. 3 A). There were endo H-sensitive high mannose oligosaccharides with 5–9 Man residues (M_5 – M_9 in the figure). In addition, there were two classes of oligosaccharides that had been extensively processed in the Golgi complex, endo H-sensitive sialylated hybrid species, and endo H-resistant complex species. This is consistent with the observation that each TfR monomer has 2–3 asparagine-linked oligosaccharides and that mature receptor has both endo H-sensitive and -resistant chains (Fig. 2 B) (40, 47).

In contrast, TfR from cells pulsed and chased in the presence of dMM had only high mannose oligosaccharides (Fig. 3 B). As expected, dMM inhibited Man trimming; high

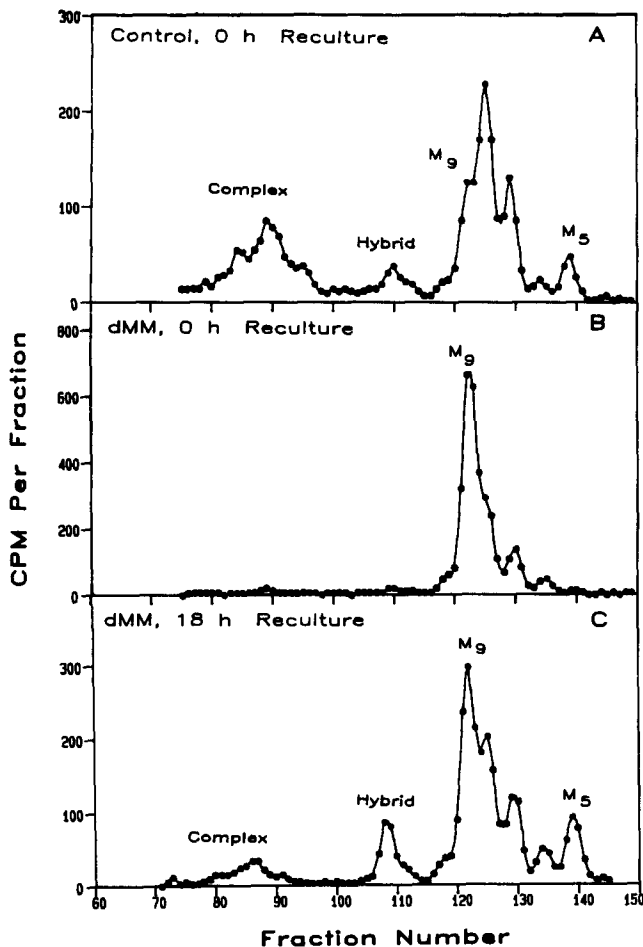


Figure 3. TfR oligosaccharides are processed during reculture after removal of dMM. Cells were pulsed with [^3H]Man for 45 min and chased for 3 h, all in the presence of 0.5 mM dMM. Cells were then washed and recultured in dMM-free medium. Control cells were treated similarly, except that no dMM was added. Samples were lysed, TfR immunoprecipitated, glycopeptides prepared, treated with endo H, and analyzed by gel filtration chromatography on Bio-Gel P-4, all as described in Materials and Methods. The elution positions of complex glycopeptides, hybrid oligosaccharides, and the high mannose species $\text{Man}_9\text{GlcNAc}$ (M_9) and $\text{Man}_5\text{GlcNAc}$ (M_5) are shown. (A) TfR from control cells at the end of the 3-h chase. (B) TfR from dMM-treated cells at the end of the chase. (C) TfR from dMM-treated cells after reculture for 18 h in dMM-free medium.

mannose oligosaccharides with 8 and 9 Man residues predominated. Moreover, because Man trimming is required for the assembly of complex and hybrid oligosaccharides, dMM blocked the synthesis of these species.

When cells were labeled in the presence of dMM and then recultured after the inhibitor was removed, significant processing of TfR^{dMM} occurred, suggesting that receptor entered the mannosidase I compartment (Fig. 3 C). After 18 h of reculture, trimmed high mannose species with 5–7 Man residues had accumulated. Moreover, substantial amounts of the sialylated hybrid and complex species were synthesized.

To quantitate these chromatographic data, the amount of radioactivity in each oligosaccharide species was determined. Data were corrected for the number of Man residues in each species and were then grouped into four categories (Fig. 4): untrimmed high mannose species ($\text{Man}_{8-9}\text{GlcNAc}_2$),

trimmed high mannose ($\text{Man}_{5-7}\text{GlcNAc}_2$), hybrid, and complex oligosaccharides. These are expressed as the percentage of total chains. The last three oligosaccharide types have all been acted on by mannosidase I.

In dMM-treated cells, untrimmed high mannose chains decreased from 70% of the total before reculture to 53% 4 h after removal of dMM and 36% 18 h after removal (compared to a value of 22% in control cells). There was a corresponding increase in trimmed high mannose, hybrid, and complex species. This process was slow, with a half-time of >6 h. The pattern of TfR oligosaccharides synthesized during the reculture of dMM-treated cells was different from that of control TfR. In recultured dMM-treated cells, there was an excess of trimmed high mannose and hybrid chains, while relatively few complex oligosaccharides were synthesized.

In control cells during the same reculture period, there was a similar maturation of oligosaccharide species (Fig. 4). The number of untrimmed high mannose species declined, with a corresponding increase in trimmed oligosaccharides. This suggests that entry into a mannosidase I-containing compartment is not a property peculiar to TfR^{dMM}, since it occurred in cells that had never been exposed to the inhibitor.

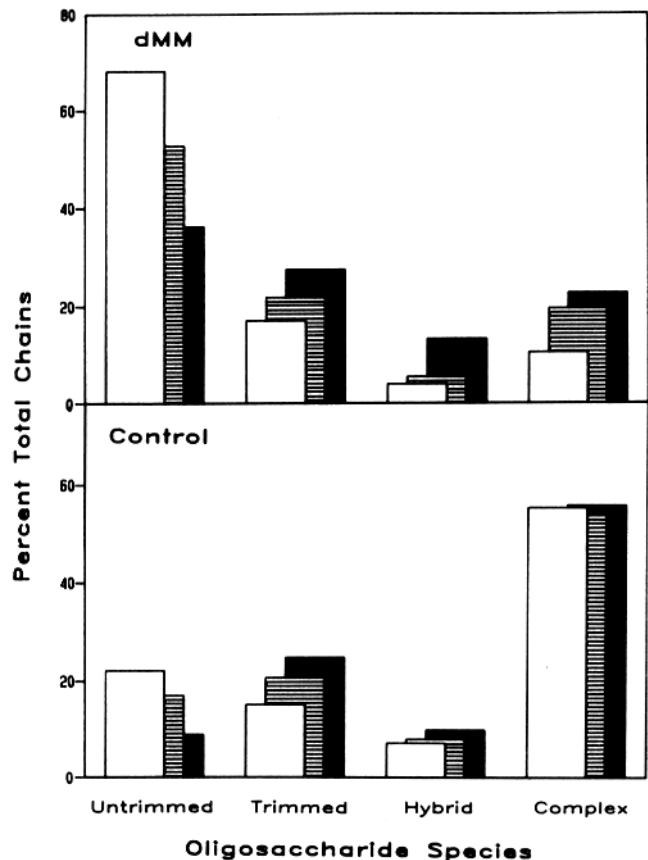


Figure 4. TfR oligosaccharides in dMM-treated and control cells. Chromatographic data from the experiment shown in Fig. 3 were quantitated by totaling the radioactivity in each peak and correcting for the number of Man residues in that species. Data are expressed as percent of total chains in four classes of oligosaccharide: untrimmed high mannose ($\text{Man}_{8-9}\text{GlcNAc}_2$); trimmed high mannose ($\text{Man}_{5-7}\text{GlcNAc}_2$); hybrid; and complex. Cells were recultured for 0 h (open bars), 4 h (hatched bars), or 18 h (solid bars) after removal of dMM.

The extent of TfR modification during reculture was significantly less in control cells than in dMM-treated cells. This is probably because TfR in control cells is nearly fully modified during transport of newly made TfR through the Golgi complex to the cell surface.

As a further test of the processing of TfR^{dMM} during reculture, the sialic acid content of the receptor was monitored. Because sialic acid is found on complex and hybrid oligosaccharides but not on high mannose species, this sugar can be used to measure the conversion of oligosaccharides to the mature forms. The addition of negatively charged sialic acid was monitored by isoelectric focusing of immunoprecipitated [³⁵S-Met]TfR (Fig. 5). TfR from cells pulsed and chased in the presence of dMM migrated as a basic species, consistent with a lack of sialic acid on receptor in these cells. After 20 h of reculture, most of the TfR^{dMM} had been converted into a more acidic form, indicating that sialic acid residues had been added. Because mannosidase I must act on TfR^{dMM} before it can be sialylated, this suggests that most of the TfR^{dMM} was first transported through the mannosidase I compartment. The receptor in recultured dMM-treated cells never became as acidic as TfR from control cells, consistent with the incomplete processing of the oligosaccharides on TfR^{dMM} (Fig. 3). The TfR species in control cells were stable during a similar period of reculture (Fig. 5).

Cell Glycoproteins Are Transported to a Mannosidase I-containing Compartment

To further study glycoprotein entry into the mannosidase I compartment, we examined the processing of total cell glycoproteins during the reculture of dMM-treated cells. Fig. 6 shows the analysis of glycoprotein oligosaccharides in the immunosupernatants from the experiment described in Figs. 3 and 4. Control cells contained a mixture of high mannose,

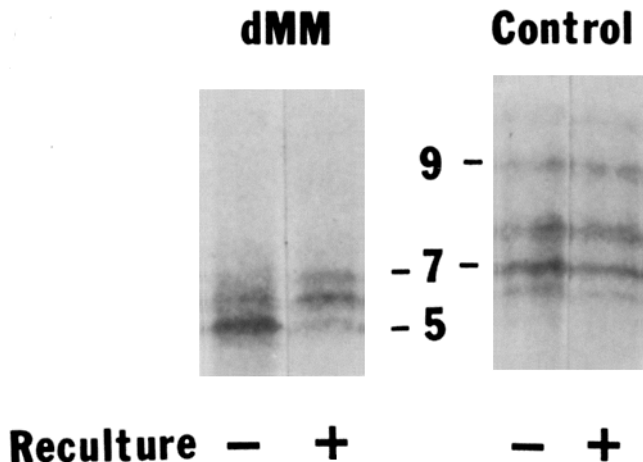


Figure 5. TfR^{dMM} is sialylated during reculture. Cells were labeled with [³⁵S]Met and chased for 2 h in the presence of dMM as described in Materials and Methods. Cells were then washed and cultured in dMM-free medium. Control cells were treated identically except that dMM was omitted. Samples were taken at the indicated times of reculture, cells lysed, and TfR was immunoprecipitated. TfR was then analyzed by slab isoelectric focusing as previously described (51). A fluorograph of the dried gel is shown with the acidic end at the top. The numbers are the ones previously assigned to the various TfR species.

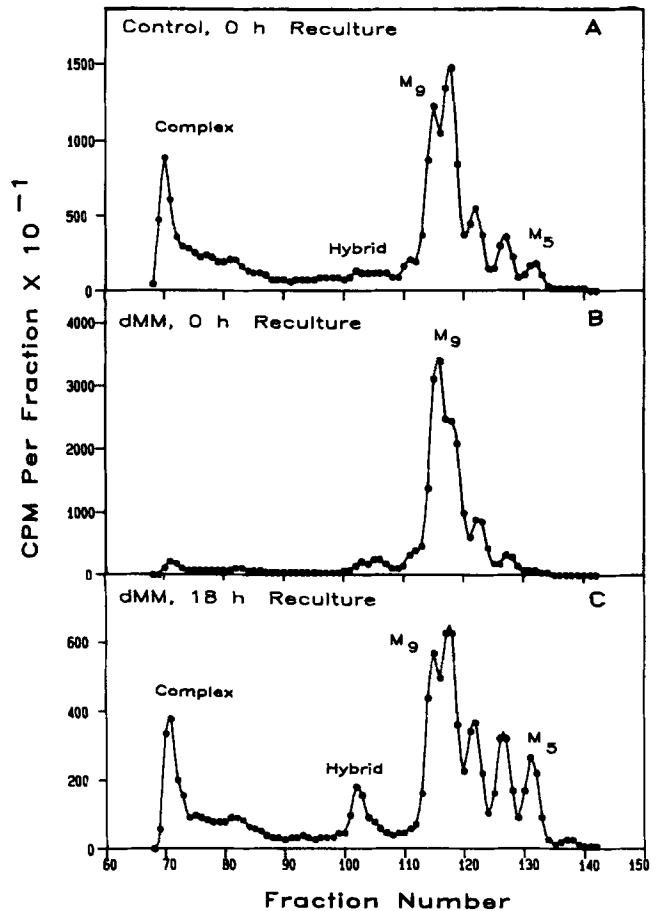


Figure 6. Total cell glycoprotein oligosaccharides are processed during reculture after removal of dMM. Cells were labeled with [³H]Man, and chased in the presence of dMM and then recultured after the dMM was removed. Total cell glycoproteins were isolated, glycopeptides prepared and analyzed as described in Fig. 3. Control cells were treated identically except that no dMM was added. The chromatograms are labeled as in Fig. 3. Glycoproteins were analyzed from (A) control cells at the end of the 3-h chase, (B) dMM-treated cells at the end of the 3-h chase, or (C) dMM-treated cells after reculture for 18 h in dMM-free medium.

hybrid, and complex species (Fig. 6 A) similar to those found on TfR.

Oligosaccharides of whole cell glycoproteins in cells pulsed and chased in the presence of dMM (Fig. 6 B) were very similar to the ones found on TfR in the same cells (Fig. 3 B). Untrimmed high mannose oligosaccharides with 8 and 9 Man residues were the predominant species. When these cells were recultured for 18 h after removal of the inhibitor, significant oligosaccharide processing occurred (Fig. 6 C), consistent with the entry of these molecules into the mannosidase I compartment. The untrimmed high mannose oligosaccharides disappeared, with a corresponding accumulation of trimmed high mannose, hybrid, and complex species. This oligosaccharide pattern is strikingly similar to the one found in control cells before reculture (Fig. 6 A).

The data for whole cell glycoprotein oligosaccharides were quantitated as described above (Fig. 7). During reculture after removal of dMM, the fraction of untrimmed high mannose chains decreased from 61% before reculture to 40% after 4 h and 24% after 18 h, (compared to 26% in control

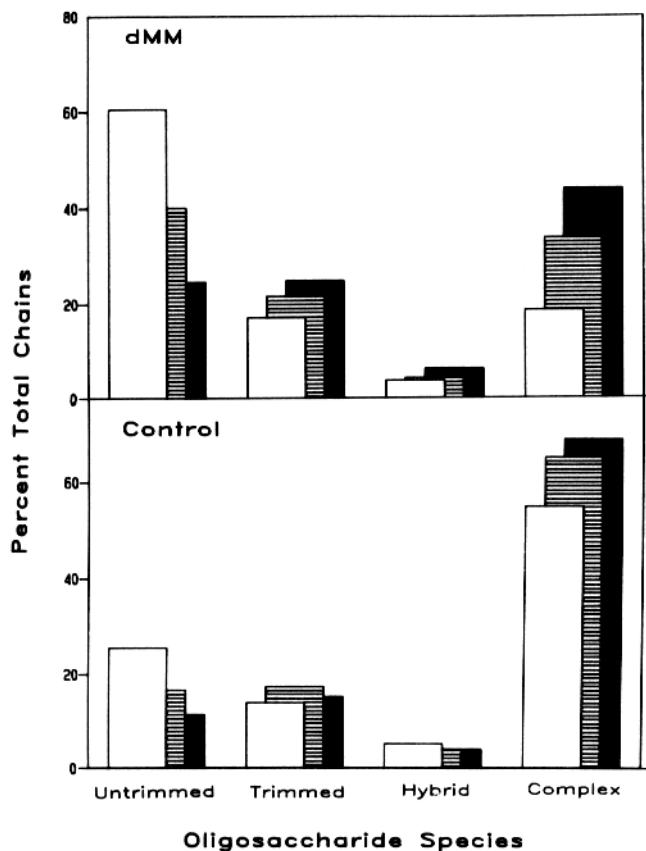


Figure 7. Total cell glycoprotein oligosaccharides in dMM-treated and control cells. Chromatographic data from the experiment shown in Fig. 6 were quantitated as described in Fig. 4. dMM-treated cells were recultured for 0 h (open bars), 4 h (hatched bars), or 18 h (solid bars) after removal of dMM.

cells). The process had a half-time of <4 h. There was a corresponding increase in trimmed high mannose and complex oligosaccharides, but little accumulation of hybrid structures was seen. As observed for TFR above, there was also continued oligosaccharide processing of whole cell glycoproteins in control cells. Untrimmed high mannose chains were converted into trimmed high mannose and complex species.

The entry of whole cell glycoproteins into mannosidase I-containing compartments was also tested in other cell types to ensure that this phenomenon is not peculiar to K562 cells. Results obtained using HeLa cells grown in suspension (Fig. 8) were similar to those described above. When dMM-treated HeLa cells were recultured for 18 h after removal of inhibitor, untrimmed high mannose oligosaccharides were processed into trimmed high mannose and complex structures. The pattern after 18 h of reculture was similar to that observed in control cells before reculture. In addition, conversion of untrimmed high mannose structures into trimmed high mannose and complex oligosaccharides was seen during the reculture of control cells. Similar results have also been obtained with Chinese hamster ovary fibroblasts and Molt-4 human leukemia cells (not shown).

Golgi Mannosidase I Trims Mannose Residues during Reculture

Mannosidases that can carry out Man trimming *in vitro* have been identified in the ER and in the Golgi complex. However,

only the Golgi enzyme is sensitive to dMM (5). To determine which of these enzymes is responsible for Man trimming during reculture, cells were pulsed with [³H]Man and chased continuously in the presence of dMM (Fig. 9). For both TFR^{dMM} and total glycoproteins, Man trimming and the synthesis of hybrid and complex species were blocked during reculture in dMM (compare to Figs. 3 C and 6 C). This suggests that the dMM-sensitive Golgi mannosidase I was responsible for the Man trimming during reculture after the inhibitor was removed.

In the total glycoprotein fraction, Man₉GlcNAc₂ was converted to Man₈GlcNAc₂ during reculture in the presence of dMM (Fig. 9). This is presumably due to the action of the dMM-resistant ER mannosidase (5). The fact that these oligosaccharides are not processed further suggests that this ER mannosidase may not act on Man₉GlcNAc₂ *in vivo*. It is also interesting to note that Man₉GlcNAc₂ appears to be quite stable on TFR^{dMM}. This may mean that TFR does not enter the compartment that contains the ER mannosidase during reculture.

In a second experiment, the inhibitor swainsonine was used to show that the observed Man trimming is due to man-

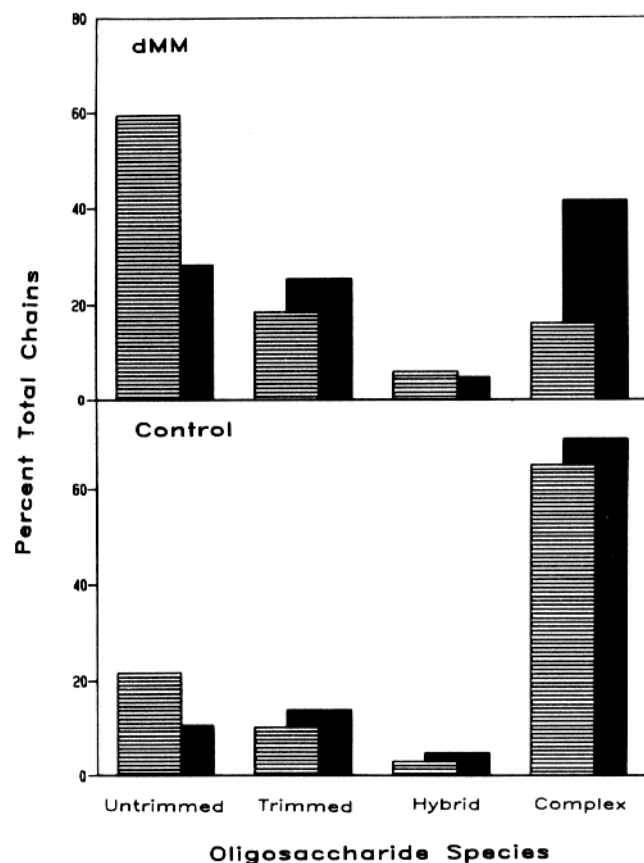


Figure 8. Total HeLa cell glycoprotein oligosaccharides are processed during reculture after removal of dMM. HeLa cells were labeled with [³H]Man and chased for 3 h in the presence of dMM. dMM was removed and the cells recultured. Total cell glycoproteins were isolated, glycopeptides prepared, treated with endo H, and analyzed by gel filtration chromatography as described in Materials and Methods. Chromatographic data, quantitated as described in Fig. 4, are expressed as the percent of total oligosaccharide chains in each species. Cells were recultured for 0 h (hatched bars) and 18 h (solid bars) after removal of dMM.

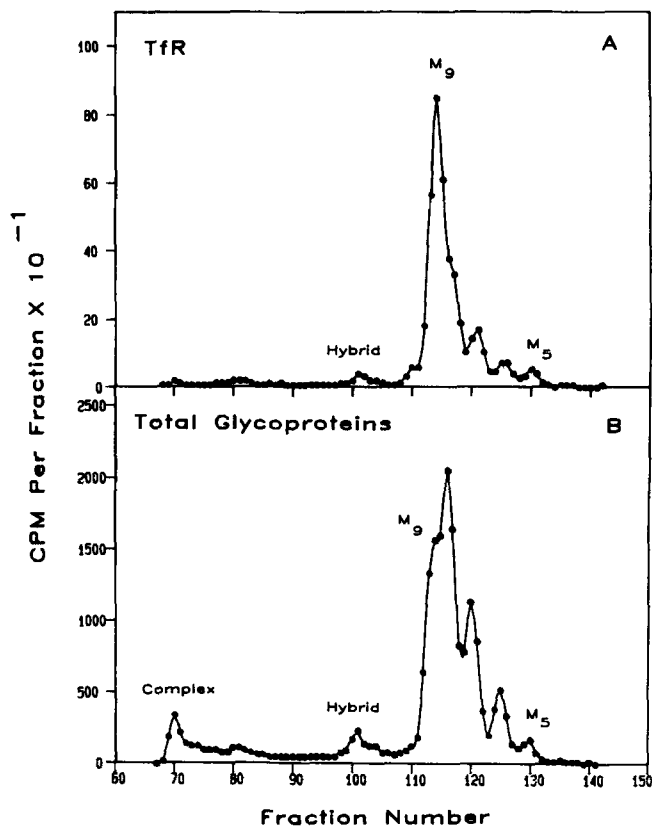


Figure 9. A dMM-sensitive mannosidase carries out Man trimming during reculture. K562 cells were labeled with [^3H]Man and chased for 3 h in the presence of dMM. Cells were then recultured for 18 h in presence of dMM, and the cells were lysed. TfR and total cell glycoproteins were then prepared and analyzed by gel filtration as described in Materials and Methods. Chromatograms are labeled as in Fig. 3.

nosidase I activity. Swainsonine does not affect Golgi mannosidase I, but inhibits both lysosomal α -mannosidase and Golgi mannosidase II (1, 12, 26, 56). The latter enzyme acts after mannosidase I during oligosaccharide synthesis and is required for the assembly of complex species (54). To study the effect of this inhibitor, cells were pulsed and chased in the presence of dMM. The dMM was then removed and the cells were recultured for 4 h with swainsonine.

Swainsonine did not affect Man trimming of either TfR or the total glycoprotein fraction (Table I), supporting the conclusion that the trimming is due to mannosidase I. However, swainsonine did inhibit complex oligosaccharide assembly during reculture, with a corresponding increase in the number of hybrid oligosaccharides synthesized (not shown). This is consistent with the role of swainsonine-sensitive Golgi mannosidase II in complex oligosaccharide synthesis.

As further confirmation that the observed Man trimming during reculture is due to the action of mannosidase I, the effects of inhibitors that block *de novo* glycoprotein synthesis were examined. Cells were pulsed and chased in the presence of dMM. The dMM was then removed and the cells recultured for 4 h in the presence of cycloheximide, which inhibits protein synthesis, or tunicamycin, which inhibits the synthesis of asparagine-linked oligosaccharides. The extent of Man trimming during reculture was not affected by either inhibitor (Table I). Thus, the changes in [^3H -Man]-oligosaccharides were not due to degradation of labeled species

and reincorporation of [^3H]Man into normal glycoproteins. This conclusion is also supported by the fact that swainsonine blocks lysosomal α -mannosidase (12). Man trimming occurred in the presence of this inhibitor (Table I), even though oligosaccharide degradation did not occur (not shown).

Mannose Trimming during Reculture Requires Intracellular Membrane Traffic

The effect of temperature on Man trimming after removal of dMM was examined to test the site where this process occurs. Reduced temperature is known to inhibit intracellular membrane traffic. At temperatures below 20°C, transport of internalized material to lysosomes (13, 37, 45), phagocytosis by macrophages (42), and export of material from the Golgi complex to the cell surface (38) are blocked.

When cells that had been pulsed and chased in the presence of dMM at 37°C were recultured without inhibitor for 4 h at 18°C, oligosaccharide trimming was almost completely blocked (Table I). This was true for the processing of both TfR and the whole cell glycoprotein fraction. These results support the idea that glycoproteins are transported into an intracellular mannosidase I-containing compartment and suggest that intracellular membrane traffic is required for this process.

A second interpretation of this result, that mannosidase I is transported to the cell surface where it acts on TfR, is unlikely. Because the cells are in steady state, inhibition of endocytic and exocytic membrane traffic by incubation at 18°C would trap mannosidase I on the cell surface. Since this enzyme is active at 18°C *in vivo* (51), this model predicts that Man trimming should occur at this temperature and can therefore be ruled out.

TfR and TfR^{dMM} Are Functionally Indistinguishable

The use of dMM to study intracellular transport of TfR is valid only if the altered oligosaccharides of TfR^{dMM} do not

Table I. Effect of Low Temperature and Inhibitors on the Processing of TfR^{dMM} and Total Glycoproteins after Removal of dMM

Treatment	Man ₈₋₉ GlcNAc ₂ (percent total chains)	
	TfR	Total Glycoproteins
Experiment I	%	%
Not recultured	65.2	60.5
Recultured, 37°C	52.7	41.6
Recultured, 37°C (1 µg/ml swainsonine)	54.8	44.0
Recultured, 18°C	63.8	58.8
Experiment II		
Not recultured	64.5	55.1
Recultured, 37°C	52.4	39.3
Recultured, 37°C (10 µg/ml cycloheximide)	53.1	42.1
Recultured, 37°C (20 µg/ml tunicamycin)	54.8	40.4

Cells were pulsed with [^3H]Man for 45 min and chased for 3 h in the presence of dMM. The dMM was then removed and the cells were recultured with the indicated inhibitors for 4 h. Cells were lysed and TfR was immunoprecipitated. Glycopeptides from TfR and total glycoproteins were prepared and analyzed, all as described in Materials and Methods. Data are expressed as the percentage of total oligosaccharide chains in the untrimmed high mannose species Man₈₋₉GlcNAc₂.

affect its function. A number of experiments were performed to show that this is the case. First, the stability of [³⁵S]Met-labeled TfR and TfR^{dMM} were compared. Both were degraded with first order kinetics at similar rates. The half-lives were 17.0 h and 19.3 h, for TfR and TfR^{dMM}, respectively. Thus the altered oligosaccharides on TfR and other glycoproteins in dMM-treated cells did not affect receptor stability.

We also compared the appearance of newly made TfR and TfR^{dMM} on the cell surface to establish that dMM does not affect the intracellular transport of TfR. Intact cells were treated with trypsin to measure surface TfR. Mild trypsinization of cells releases a water-soluble TfR fragment with *M_r* 70,000 (40, 47). Therefore, surface TfR can be measured as the fraction of receptor lost from the cells on protease treatment.

In cells pulse-labeled with [³⁵S]Met, only 7% of the labeled TfR was protease sensitive. Since all of the molecules should be within the cells at this time, this shows that protease was not penetrating the cells. When cells that had been pulsed and chased for 2 h were analyzed, 35% of the labeled TfR was found on the cell surface, while 32% of the labeled TfR^{dMM} was found on the surface. This demonstrates that TfR^{dMM} is transported to the cell surface at a rate similar to that of TfR in control cells. This is the fraction of receptor found on the surface by other workers using surface proteolysis (6, 59), and the distribution of transferrin binding sites (10, 34). We also obtained similar results using neuraminidase treatment of intact cells (51) to probe for surface receptor (not shown).

Transferrin binding and internalization by TfR and TfR^{dMM} were also compared. Cells were incubated with ¹²⁵I-transferrin at 0°C to saturate surface binding sites. They were then washed and incubated in unlabeled medium at 37°C. The total, internal, and surface cell-associated transferrin were measured, as well as transferrin released into the medium. To study TfR^{dMM}, cells treated with the inhibitor for 24 h were used. Roughly, two-thirds of the surface TfR in these cells is TfR^{dMM}, which was demonstrated by iodinating the cell surface and then immunoprecipitating and analyzing the labeled TfR by SDS gel electrophoresis (not shown).

The binding and internalization of transferrin by TfR and TfR^{dMM} were nearly identical (Fig. 10). Transferrin that was bound to surface receptors was rapidly internalized and then released intact into the medium. Most of the bound transferrin had been internalized within 5 min, while 85–90% was released after 30 min of incubation. This experiment shows that the altered oligosaccharides of TfR^{dMM} do not affect its ability to bind, internalize, and release its ligand transferrin.

Finally, we showed that dMM did not affect cell growth or macromolecular synthesis. The growth rates of cells treated with dMM for 4 h were identical to controls and >98% of the cells in both cultures excluded trypan blue. In addition, rates of DNA and protein synthesis, measured by the incorporation of [³H]thymidine and [³⁵S]Met into acid-precipitable material were the same in treated and control cultures.

Previous studies have also shown that dMM does not affect intracellular membrane traffic or glycoprotein function. Burke et al. (8) have shown that dMM does not affect the arrival of newly made glycoproteins at the cell surface.

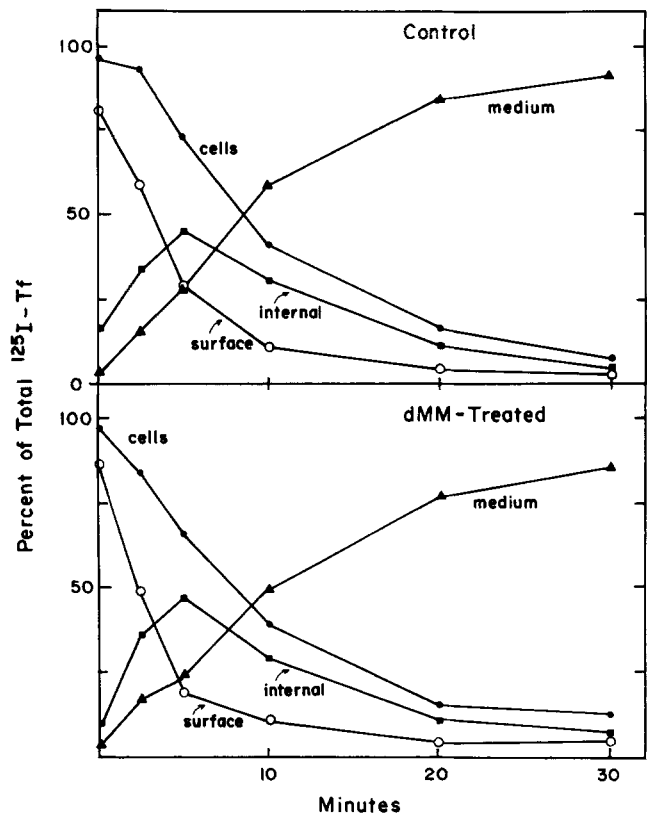


Figure 10. Endocytosis and release of transferrin are similar in control and dMM-treated cells. K562 cells were cultured for 24 h in 0.5 mM dMM. These cells and control cells were incubated with ¹²⁵I-transferrin at 0°C, washed, and then incubated at 37°C. Transferrin that was cell associated, surface, internal, or free in the medium was determined as described in Materials and Methods.

Moreover, the production of several enveloped viruses is normal in dMM-treated cells (8, 17).

Discussion

In this study, we have examined the entry of cellular glycoproteins into the compartments where Man trimming occurs. Mannosidases that carry out this trimming have been identified in both the ER (2, 4) and the Golgi complex (20, 55, 57). However, Bischoff and Kornfeld (5) showed that only Golgi mannosidase I is sensitive to dMM. Because we found that nearly all the Man trimming activity was dMM sensitive, our experiments have measured glycoprotein transport into the compartment that contains Golgi mannosidase I.

The exact location of mannosidase I in the Golgi complex is not clear. In cell fractionation experiments, Dunphy and Rothman showed that mannosidase I is resolved from the *trans* Golgi enzyme galactosyltransferase but fractionates with the medial Golgi enzyme GlcNAc transferase I (14–16). In addition, it has been shown that newly made glycoproteins move through the Golgi complex in a *cis*-to-*trans* direction (3, 46), and that mannosidase I must act before GlcNAc transferase I during oligosaccharide processing (35, 50). Taken together, these results are consistent with the location of mannosidase I in either a *cis* or a medial Golgi region.

Transferrin Receptor Returns to the Mannosidase I Compartment

We find that TFR^{dMM} is transported into a mannosidase I-containing compartment, suggesting that it returns to this region of the Golgi complex. Since most of the TFR^{dMM} molecules entered this compartment during 20 h of reculture (Fig. 5), it is likely that these receptors are actively involved in transferrin endocytosis and include the cell surface pool. The processing is quite slow, with a half-time of at least 6 h. However, this is a minimum estimate of the transport rate. If processing is inefficient, then several exposures to mannosidase I might be required before oligosaccharide processing occurred and transport would be faster than the observed rate of Man trimming.

During the reculture of dMM-treated cells, sialylated hybrid and complex oligosaccharides were synthesized on TFR^{dMM}. The synthesis of these structures requires GlcNAc transferase I, a medial Golgi enzyme (15), and sialyltransferase, a *trans* Golgi enzyme (43, 44). Because these processing enzymes must act in a well-defined sequence, this suggests that TFR molecules follow the normal exocytic pathway through the Golgi complex after entering the mannosidase I compartment. However, we cannot rule out the possibility that the observed oligosaccharide processing occurs in a non-Golgi location.

While mature oligosaccharides are synthesized on TFR^{dMM} during reculture, the pattern is different from control TFR. TFR^{dMM} in recultured cells has an excess of hybrid oligosaccharides and fewer complex species relative to the oligosaccharides found on TFR in normal cells. This could be caused by differences between newly made TFR and recycled TFR^{dMM}. The differences could be in the conformation of the receptor polypeptide or in its posttranslational modification. For example, fatty acyl (40) and phosphate residues (47) found on mature TFR may not be present during the processing of newly made receptor.

We have previously used the approach described in this study to examine the entry of TFR into compartments that contain sialyltransferase, an enzyme that acts late in oligosaccharide modification. Sialyltransferase has been localized to the *trans* region of the Golgi complex (43, 44) and has been resolved from mannosidase I in cell fractionation experiments (14, 16). We found that asialo-TFR generated on the cell surface is resialylated (51), suggesting that it is transported to the site of sialyltransferase in the Golgi complex. The half-time of resialylation is 2–3 h, significantly faster than the Man trimming of TFR^{dMM}. Thus, TFR appears to enter the sialyltransferase compartment in the Golgi complex faster than it enters the mannosidase I compartment. This difference could be due to a greater rate of vesicular traffic into the sialyltransferase compartment or could be caused by preferential uptake of TFR into vesicles destined for that region.

While we have shown that TFR moves into both the mannosidase I and sialyltransferase compartments, the importance of this transport is not clear. Transferrin bound to surface TFR is internalized, returns to the cell surface, and is released into the medium within 10–20 min (Fig. 10) (11, 32, 33, 39). Therefore it is unlikely that a receptor enters enzyme-containing compartments in the Golgi complex each time it is internalized. Rather, a small fraction of the internal-

ized TFR is transported to the Golgi compartments, with all receptors following this pathway eventually.

Total Cell Glycoproteins Return to the Mannosidase I Compartment

The total cell N-linked glycoprotein pool also enters the mannosidase I compartment. This argues that many cellular glycoproteins return to this region of the Golgi complex. The half-time for Man trimming on these molecules is <4 h, which is faster than the trimming of TFR^{dMM}. The Golgi complex makes up roughly 5% of the glycoprotein-containing membranes in mammalian cells, based on morphometric data from several cell types (7, 9, 27). This finding, combined with the fact that Man trimming did not occur when intracellular membrane traffic was blocked, suggests that the trimmed glycoproteins are transported into the mannosidase I compartment during reculture.

Not only does Man trimming occur during the reculture of dMM-treated cells, but the oligosaccharides synthesized are similar to those in control cells. This suggests that glycoproteins which are normally processed by mannosidase I as newly made molecules probably recycle through the mannosidase I compartment. Similarly, the glycoproteins that are not processed as newly made molecules are not processed during reculture, either because they never enter the mannosidase I compartment, or because they are not substrates for the enzyme. The glycoproteins that are processed during reculture acquire mature hybrid and complex oligosaccharides. Thus, these molecules probably follow the normal exocytic pathway from the mannosidase I compartment, since they must be transported through the Golgi complex, where they are exposed to later-acting processing enzymes.

There is other evidence supporting a slow exchange of membrane constituents between the cell surface and Golgi complex. First, electron microscopic studies have shown that surface TFR is transported to Golgi cisternae (60). Nonspecific tracers bound to the plasma membrane are also transported into Golgi cisternae. Markers were found in all cisternae, although labeling was heaviest in the *trans* region (18, 25, 28, 29, 30, 41). Second, incubation of cells with antibody to the mannose-6-phosphate receptor resulted in secretion of newly made lysosomal enzymes. This is consistent with mixing of cell surface and Golgi pools of receptor, or with the uptake of extracellular antibody into the Golgi complex (22). Third, immunoelectron microscopy has localized a substantial fraction of asialoglycoprotein receptors to the Golgi complex in hepatocytes (23, 58). While it has been shown that these are not newly made molecules on their way to the cell surface, the ability of these receptors to mix with the surface pool has not been tested. Finally, Sleight and Pagano (49) have found that a fluorescent phospholipid in the plasma membrane of cultured fibroblasts is transported into the Golgi complex.

The finding that the total N-linked glycoprotein pool recycles through the mannosidase I compartment has important implications for the nature of membrane traffic through the Golgi complex. This transport has a half-time of <4 h. In contrast, most glycoproteins have half-lives that are considerably longer. In K562 cells, the half-life of these glycoproteins is ~25 h (not shown). This suggests that a glycoprotein recycles through the mannosidase I compartment 5–10

times during its lifetime. Moreover, this means that most of the glycoprotein traffic through this compartment in the Golgi complex is made up of recycling material. For every newly made glycoprotein that enters this compartment, 5–10 recycling molecules enter.

The importance of this membrane traffic is not clear. It is possible that the recycling is a retrieval mechanism. Membrane traffic from the Golgi complex to the cell surface carries newly made membrane and secretory constituents. The recycling of glycoproteins may represent a way of balancing the flow out of the Golgi complex. A second possibility is that the recycling represents a mechanism that allows post-translational modifications to be continuously repaired. Consistent with this finding is the observation that fatty acyl residues are added to both old and new TfR molecules (40). This recycling could also allow posttranslational modifications to be changed on pre-existing proteins in response to physiological stimuli. We are hopeful that closer examination of recycling through the mannosidase I compartment, as well as similar studies of transport through other enzyme-containing compartments in the Golgi complex, will reveal the importance of the relationship between exocytic and endocytic membrane traffic.

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