Recycling Glycoproteins Do Not Return to the *cis*-Golgi

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Abstract. Recycling of a number of glycoproteins along the site of action of mannosidase I (the distal endoplasmic reticulum/*cis*-Golgi) was followed in several different cell lines. Treatment of cells with 1-deoxymannojirimycin (dMM) produced high mannose oligosaccharides at positions otherwise occupied by complex-type oligosaccharides in these glycoproteins. Conversion of high-mannose-type oligosaccharides to complex oligosaccharides of proteins initially synthesized in the presence of dMM was used as a marker for recycling of glycoproteins along the site of action of dMM. In contrast to findings reported by Snider and Rogers (Snider, M. D., and O. C. Rogers. 1986. *J. Cell Biol.* 103:265-275), removal of dMM did not result in reconversion of high-mannose oligosaccharides to complex-type sugars, even after prolonged periods of culture. We conclude that surface glycoproteins do not recycle through the *cis*-medial Golgi elements.

ENDOCYTOSIS of surface receptors is one of the mechanisms by which the cell controls uptake of nutrients, and levels of surface receptors. Any stimulus delivered by hormones or growth factors may likewise be dependent on, or modulated by receptor-mediated endocytosis (7, 8). The endocytotic pathway has been unraveled in considerable detail morphologically and biochemically (17, 26). Far less is known about the route taken by recycling receptors on their way back to the cell surface, because it is difficult to discriminate between newly synthesized receptors that are on their way to the plasma membrane, and recycling receptors that were already resident at the cell surface.

In principle, the exocytotic aspect of the recycling pathway can be studied, provided the following conditions can be fulfilled. Receptors resident at the cell surface need to be tagged specifically; the label used should not perturb the recycling process per se; traversing a particular subcellular compartment should produce a detectable change in the tagged receptor. Lactoperoxidase-catalyzed iodination of surface receptors in conjunction with neuraminidase digestion has been used to produce labeled, desialylated surface molecules that can report on traffic through the *trans*-Golgi (22). Under these circumstances, acquisition of sialic acid(s) is interpreted as indicative of traversal through the *trans*-Golgi network.

A second approach is to use modifications of N-linked glycans as produced by oligosaccharide-trimming inhibitors (6). Use of the mannosidase I inhibitor 1-deoxymannojirimycin (dMM)[1] results in retention of high-mannose oligosaccharides for those glycans normally converted to the complex type (5). In a pulse-chase experiment, surface receptors carrying high-mannose glycans as a consequence of treatment with dMM can be delivered to the cell surface in a biosynthetically labeled form. If inhibition by dMM is reversible, then these high-mannose glycans can report on recycling pathways involving the compartment which is the target of inhibition of dMM, i.e., the distal endoplasmic reticulum (ER) and *cis*-Golgi (1, 5).

Experiments of this type were performed by Snider and Rogers (24), who concluded that recycling molecules returned to the site of inhibitory action of dMM. These studies were performed on the transferrin receptor (Tfr) in the erythroleukemic cell line K562.

We are unable to confirm these findings for the Tfr not only in K562 cells, but also in the human hepatoma cell line HepG2. Moreover, class I antigens present on HepG2 and lymphoblastoid cell lines do not convert their high-mannose glycans to complex-type glycans in experiments of this type, nor do class II molecules in lymphoid cells.

We conclude that, within the limits of resolution of these experimental systems, recycling of membrane proteins through the distal ER and *cis*-medial Golgi is insignificant.

Materials and Methods

Cells and Cell Culture

Human hepatoma cells (HepG2) were grown in DME supplemented with 10% FCS. Human lymphoblastoid cells (JY) and human erythroleukemia cells (K562) were grown in suspension in RPMI-1640 medium supplemented with 10% FCS.

Antibodies

The following antibodies were used. Rabbit anti-human α_1-antitrypsin;

1. *Abbreviations used in this paper*: dMM, 1-deoxymannojirimycin; Endo H, endoglycosidase H; ER, endoplasmic reticulum; FPLC, fast protein liquid chromatography; NANAse, neuraminidase; Tf, transferrin; Tfr, transferrin receptor; Tfr^{dMM}, transferrin receptor synthesized in the presence of dMM.

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rabbit anti-human transferrin (Central Laboratory of the Blood Transfusion Service, Amsterdam); mouse anti-human transferrin receptor (Mab 66 Ig 10) (29); W6/32 (a mouse Mab recognizing HLA-A, -B, and -C locus products) (16); and a rabbit anti-class II serum (13).

Gel Electrophoresis

SDS gel electrophoresis (11) and one dimensional isoelectric focussing (one-dimensional IEF) were performed as described (12). Immunoprecipitations were performed as described (3).

Enzymatic Digestions and Glycosylation Inhibitors

The immunoprecipitates were resuspended in 10 μl digestion buffer (50 mM Na-citrate pH 5.5) containing 0.2% SDS and heated to 95°C for 5 min. After cooling, 2 mU endoglycosidase H (Endo H) (Boehringer Mannheim Diagnostics, Inc., Houston, TX) in 10 μl digestion buffer was added. Digestion was carried out at 37°C for 20 h. The reaction was stopped by addition of SDS sample buffer.

Digestions with neuraminidase type VIII (Sigma Chemical Co., St. Louis, MO) were performed as described (12).

dMM was synthesized using a new synthetic scheme (2) and was used as described (5) at a concentration of 1 mM.

Reversibility of Mannosidase I Inhibition by dMM in HepG2 Cells

HepG2 cells were grown to subconfluency in 60-mm petri dishes in DME + 10% FCS. Before labeling, cells were incubated for 30 min in methionine-free RPMI medium.

To determine the time required for dMM to fully exert its inhibitory effect, dMM (1 mM) was added to the culture medium (2 ml of medium per 60-mm dish), followed by metabolic labeling with 25 μCi [^{35}S]methionine per dish at 0, 60, and 300 min after addition of dMM. HepG2 cells were labeled for 20 min and chased for 30 min by addition of nonradioactive methionine to a final concentration of 1 mM. Control cells were labeled with 25 μCi [^{35}S]methionine for 20 min, followed by a 30-min chase. α_1-antitrypsin was then immunoprecipitated from the culture medium.

To establish the reversibility of inhibition by dMM, dMM (1 mM) was added to the culture medium for 5 h. HepG2 cells were transferred to methionine-free medium (but also containing dMM) 30 min before removal of dMM. To remove dMM, cells were washed three times with PBS at 37°C. Cells were again transferred to methionine-free medium, pulsed with 25 μCi [^{35}S]methionine for 20 min, and chased for 30 min. α_1-antitrypsin was then immunoprecipitated from the culture medium.

Recycling Experiment on HepG2 Cells

HepG2 cells were grown to subconfluency in 60-mm petri dishes in DME + 10% FCS. Each dish was labeled in methionine-free RPMI medium with 25 μCi of [^{35}S]methionine for 5 h after the addition of dMM (1 mM). The incorporation of label was stopped by the addition of nonradioactive methionine to a final concentration of 1 mM and the cells were chased for 1 h. Each dish was washed three times with PBS at 37°C and incubation was continued in DME + 10% FCS. At different timepoints (1, 3, 6, 12, and 24 h) after dMM removal a dish was taken, cells were lysed in NP-40-containing lysis buffer (11), and Tfr as well as class I antigens were immunoprecipitated as described (3).

To verify that inhibition of mannosidase I activity by dMM was complete, one dish was taken immediately before dMM removal. Cells were lysed with NP-40 lysis mix and Tfr and class I antigens were immunoprecipitated.

To examine Tfr and class I antigens in control cells, one dish was labeled with 25 μCi of [^{35}S]methionine for 5 h in the absence of inhibitor and chased for 1 h. Cells were lysed with NP-40 lysis buffer and Tfr and class I were immunoprecipitated.

Reversibility of Mannosidase I Inhibition by dMM in K562 Cells

K562 cells were maintained in RPMI + 10% FCS. Cells were labeled for 5 h in methionine-free RPMI 1640 + 10% FCS at a density of 5.10^6 cells/ml, using 25 μCi [^{35}S]methionine/ml. Incorporation of label was stopped by addition of nonradioactive methionine to a final concentration of 1 mM, and incubation was continued (chase) for 60 min. For each chase time, aliquots of 1 ml (5.10^6 cells) were taken. The experiment was otherwise performed as described for HepG2 cells. Cells were lysed in 1 ml NP-40-containing lysis buffer (12) and Tfr was immunoprecipitated.

Recycling Experiments on K562 Cells

5.10^6 K562 cells were used for each condition and were labeled with 25 μCi [^{35}S]methionine. The experiment was performed as described for HepG2 cells, except that incubation after dMM removal was continued in RPMI + 10% FCS.

Recycling Experiments JY Lymphoblastoid Cells

Recycling experiments involving JY cells were performed as described for K562 cells. Class I and class II antigens were immunoprecipitated sequentially as described (13).

Recycling of ^{125}I-Tf in HepG2 Cells

Human transferrin Tf (T-2252; Sigma Chemical Co.) was iodinated to a specific activity of 9 μCi/μg using chloramine T iodination (9).

HepG2 cells were grown in 60-mm tissue culture dishes to subconfluency. For dMM treatment, cells were cultured for 72 h in the presence of 1 mM dMM. Control cells and dMM-treated cells were transferred to 0°C and ^{125}I-Tf was added (3 μg per dish for 10 min). Incubation was continued on ice (resulting in binding, but not internalization, of Tf [10]) or at 37°C (resulting in binding and uptake of ^{125}I-Tf). To stop internalization, cells were transferred to 0°C. Unbound ^{125}I-Tf was removed by washing the cells three times with PBS at 0°C. Subsequently, surface bound ^{125}I-Tf was removed by exposing the cells to a pH shock for 10–15 s with 1 ml 0.25 M HAc/0.5 M NaCl pH 2.3. The cells were immediately neutralized with 1 ml 1 M NaAc and washed twice with PBS at 0°C. 5 ml DME was then added to each dish. The protocol described here for removal of surface Tf is essentially as described in reference 10.

Incubation was then continued at 37°C, where recycling should result in the release of ^{125}I-apotransferrin, or at 0°C, where release of label is not due to recycling, but is nonspecific (i.e., due to lysis of and leakage from cells). 1-ml samples were taken from the supernatant at different time points (t = 0, 2.5, 5, 15, 30 min). The amount of released ^{125}I-Tf in the medium was measured by gamma counting. The condition where binding of ^{125}I-Tf was allowed at 0°C, followed by removal of surface bound ^{125}I-Tf, and incubation continued at 0°C represents the background of such an experiment (Fig. 2, curve labeled *0,0°C*).

When binding and uptake were allowed at 37°C (Fig. 2, curve *37, 37°C*) or 0°C (curve *37, 0°C*), the difference between these two curves represents the amount of ^{125}I-Tf released due to recycling. This experiment was carried out for control and dMM-treated cells. To verfiy that in dMM-treated cells all Tfr had the predicted type of N-linked glycan, Tfr was isolated from lactoperoxydase surface labeled (19) control and dMM-treated cells, and analyzed by SDS-PAGE before and after Endo H digestion.

Labeling and Analysis of Total N-linked Carbohydrates

Labeling and analysis of total N-linked carbohydrates was performed essentially as described by Snider and Rogers (24). Briefly, 4.10^7 K562 cells were labeled with 300 μCi 2[^{3}H]mannose for 45 min in either the presence or the absence of dMM in RPMI containing 0.1 mg/ml glucose and 5% FCS. Cells were chased for 3 h through addition of normal RPMI + 10% FCS. Cells were washed three times with PBS in order to remove dMM, as described above.

A sample of $1,3.10^7$ K562 cells was taken at timepoint 0′ after chase, and after 18 h reculture in RPMI + 10% FCS as well as in RPMI + 10% FCS supplemented with 20 mM mannose to prevent reutilization of radioactive mannose. Cells were lysed in 1 ml NP-40 lysis buffer. Total lysates were digested with 5 mg of pronase per ml of lysate at 37°C. Every 2 h, 5 mg of pronase per ml of lysate was added and this was repeated six times. Digested material was dialyzed (pore size of dialysis membranes 800 D) and samples were analyzed on a Mono Q column coupled to fast protein liquid chromatography (FPLC) equipment (LKB Instruments, Inc., Gaithersberg, MD) as described (28). The radioactivity in the samples was determined by liquid spectrometry.

Finally, 21.000 cpm were obtained from control cells at 0 h. After 18 h of reculture or 18 h of reculture in the presence of 20 mM mannose, 55 and 50%, respectively, of the label was recovered. From dMM-treated cells 7,700 cpm was recovered at the 0-h timepoint. After 18 h of reculture or 18 h of reculture in the presence of 20 mM mannose 58 and 49%, respectively, of the label was recovered. As judged from the amount of radioactiv-

ity recovered, dMM apparently inhibits uptake/incorporation of radiolabeled mannose.

We have not observed any inhibitory effects of dMM on incorporation of [^{35}S]methionine for the three cell lines used in this study.

Results

To establish whether glycoproteins recycle under physiological conditions along the compartment(s) in which mannosidase I is located (the endoplasmic reticulum/*cis*-Golgi) (1, 4), the following protocol was followed. Cells were labeled with [^{35}S]methionine for 5 h in the presence of the mannosidase I inhibitor dMM and chased for 1 h in order to allow passage of the radiolabeled proteins along the site of action of mannosidase I. The inhibitor dMM was removed by washing, resulting in a reversal of the inhibition imposed by dMM, as will be shown below. Thus, all proteins passing the site of action of mannosidase I should now obtain their normal N-linked carbohydrate type. After removal of dMM, cells were analyzed at different timepoints, and the conversion of high-mannose to complex type N-linked carbohydrate chains on the radiolabeled proteins was followed in time by glycosidase digestions and analysis by isoelectric focussing. Reconversion of high-mannose to complex-type N-linked carbohydrates can only occur through mannosidase I activity, followed by the action of GlcNAc transferase I and α-mannosidase II (6), and thus would prove recycling through the *cis*-medial-Golgi.

Inhibition by dMM Is Rapid and Reversible

To perform the experiment as described above, we examined the reversibility of inhibition by dMM. HepG2 cells were labeled for 10 min after addition or removal of dMM at timepoints as indicated in Fig. 1 and chased for 30 min. α_1-antitrypsin was precipitated from the medium and half of the immunoprecipitates were treated with Endo H. Inhibition of mannosidase I by dMM results in high-mannose carbohydrates, and thus Endo H sensitivity of α_1-antitrypsin. α_1-antitrypsin normally carries complex type N-linked sugars and is Endo H resistant (Fig. 1 *A*, lanes *2* and *3*).

Fig. 1 *a* shows that dMM inhibited mannosidase I activity immediately after its addition (lanes *4* and *5*). The inhibitory effect was reversible within 30 min after removal of dMM by washing (Fig. 1 *a*, lanes *10* and *11*). To analyze this phenomenon for the Tfr, K562 cells were pulsed for 20 min and chased for 60 min (see Fig. 1 *b*). For K562 cells similar times of action of dMM were observed as for HepG2. For K562 also, this inhibition is reversible within 30 min. Note that Tfr is partly Endo H sensitive, in agreement with the observation that Tfr contains both high-mannose and complex N-linked carbohydrates (14, 15, 25). Reversibility of the inhibitory action of dMM was likewise established for the JY cell line, and was strictly comparable to our observations for K562 and HepG2 (results not shown). Our data are in agreement with those of Snider and Rogers (24).

dMM Does Not Affect Binding of Tf to Tfr and Recycling of Tfr

We established that changing the carbohydrate type by dMM treatment did not affect receptor recycling. We carried out recycling experiments for the HepG2 cell line based on published protocols as described in detail in Materials and Methods. Fig. 2 *a* shows that HepG2 cells, after uptake of

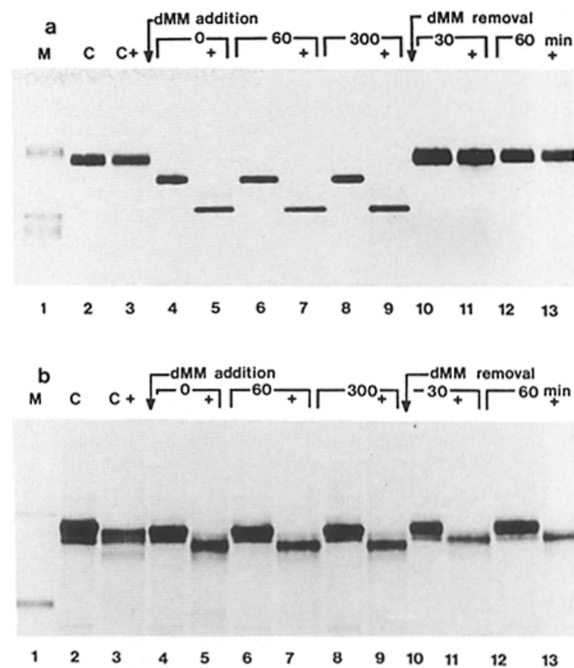

Figure 1. Inhibition by dMM is reversible. Cells were exposed to dMM (1 mM final concentration) and labeled at various timepoints after exposure to dMM. Reversal of inhibition was measured by removal of inhibitor by washing, and labeling at 30 and 60 min after removal. (*a*) HepG2 cells were exposed to 1 mM dMM and labeled for 10 min with [^{35}S]methionine at t = 0 (lanes *4, 5*), t = 60 (lanes *6, 7*), and t = 300 min (lanes *8, 9*) after addition of dMM. HepG2 cells were also labeled in the absence of dMM (lanes *2, 3*). After a 5-h exposure to dMM, the inhibitor was removed by washing, and cells were labeled (for 10 min) at 30 (lanes *10, 11*) or 60 min (lanes *12, 13*) after dMM removal. Cells were chased for 30 min, and α_1-antitrypsin was immunoprecipitated from the medium. Half of the immunoprecipitates were treated with Endo H (lanes indicated by +). Analysis was by SDS-PAGE (10% acrylamide). Control cells (lanes *2* and *3*) were labeled and chased in the absence of dMM. *M*, marker proteins: 92, 68, and 45 kD. (*b*) K562 cells were exposed to 1 mM dMM and labeled for 20 min with [^{35}S]methionine at t = 0 (lanes *4, 5*), t = 60 (lanes *6, 7*), and t = 300 min (lanes *8, 9*) after exposure to dMM. K562 cells were also labeled in the absence of dMM (lanes *2, 3*). Cells were chased for 60 min. Tfr was immunoprecipitated and analyzed by Endo H digestion (lanes indicated by +) followed by analysis on SDS-PAGE (6% acrylamide). After a 5-h exposure to dMM, the inhibitor was removed by washing, and cells were labeled and chased for 20 and 60 min (lanes *10, 11*), respectively, at 30 and 60 min (lanes *12, 13*) after dMM removal. *M*, marker proteins: 92 and 68 kD, respectively.

^{125}I-Tf for 10 min at 37°C, released Tf only at 37°C and not at 0°C. The kinetics of release was indistinguishable for cells grown in the presence of dMM for 3 d and control cells. There was no difference in binding of ^{125}I-Tf to Tfr^{dMM} and Tfr, as measured on intact cells at 0°C (data not shown). To show that dMM had indeed quantitatively changed the carbohydrate type of the Tfr, a biochemical analysis of surface-labeled Tfr was carried out and is shown in Fig. 2 *b*. HepG2 cells cultured in the presence or absence of dMM for 3 d were surface iodinated. The Tfr was immunoprecipitated, digested with Endo H, and analyzed by SDS-PAGE. The Tfr^{dMM} had a lower apparent molecular mass than the Tfr,

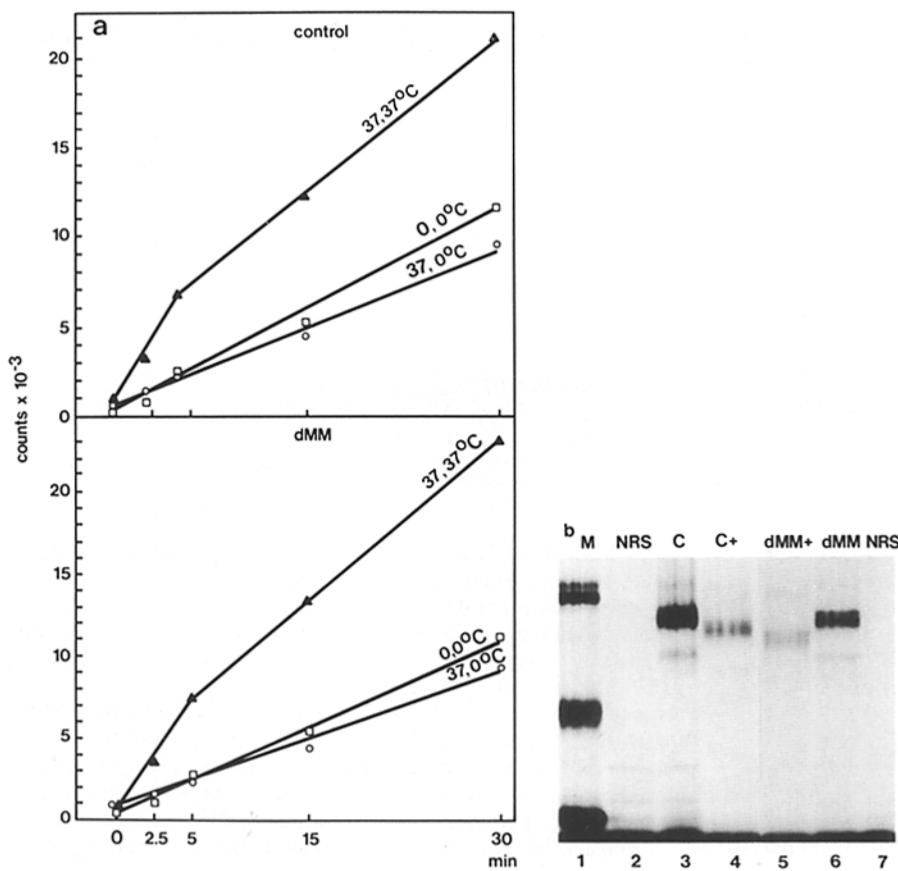

Figure 2. (*a*) Release of ^{125}I-transferrin by control and dMM-treated HepG2 cells. HepG2 cells were incubated with ^{125}I-Tf at 37 or 0°C for 10 min, and then transferred to 0°C. Surface-bound ^{125}I-Tf was removed by exposure to low pH at 0°C (10), and the low pH buffer was then replaced with ice-cold fresh medium. Incubation was then continued on ice, or at 37°C, and released ^{125}I-Tf was measured. The first number over the curve indicates the temperature at which exposure to ^{125}I-Tf took place, the second number the temperature at which release was measured. Specific release of ^{125}I-Tf takes place at 37°C only, and is similar in kinetics and quantity for control cells (*top*) or cells cultured for 3 d in dMM (*bottom*). (*b*) All Tfr that can be surface labeled in dMM-treated HepG2 cells carries high-mannose N-linked oligosaccharides only. Equal amounts of HepG2 cells (lanes *3* and *4*) and HepG2 cells cultured in the presence of 1 mM dMM for 72 h (lanes *5* and *6*) were cell surface iodinated. The Tfr was precipitated and treated with (+) or without Endo H. Samples were analyzed on a 6% SDS-PAGE. Lane *1*, molecular mass markers. Normal rabbit serum control (*NRS*) from control cells (lane *2*) as well as for dMM-treated HepG2 cells (lane *7*) are shown. Surface-labeled Tfr from dMM-treated cells is completely Endo H sensitive, resulting in a lower molecular mass after digestion than similarly treated Tfr from control cells. *M*, marker lane: 92, 68, and 45 kD, respectively.

showing that 3 d of culture in the presence of dMM had changed the carbohydrate type of all Tfr that could be surface labeled.

Having established that the inhibitory effect of dMM is reversible, and that dMM treatment did not affect recycling of the Tfr in K562 and HepG2 cells, the recycling of glycoproteins along the site of Mannosidase I (the ER/*cis*-Golgi) (1, 4) was followed as described in Materials and Methods. Three different cell lines were used: the cell line K562 (Tfr), the hepatoma cell line HepG2 (Tfr and class I antigens), and the Epstein-Barr virus–transformed cell line JY (class I and class II antigens).

Do Transferrin Receptors in K562 Cells Return to the Site of Action of Mannosidase I?

K562 cells, labeled for 5 h in the presence of dMM, and recultured for various periods of time in the absence of dMM, were used as a source of Tfr. The immunoprecipitated Tfr were analyzed after digestion with Endo H on SDS-PAGE. Equal amounts of radioactive, immunoprecipitated, Tfr were loaded on gel. Tfr from control cells is partially Endo H sensitive, because it contains two high-mannose and one complex-type N-linked oligosaccharide (13, 14, 24). Tfr^{dMM} contains only high-mannose N-linked oligosaccharides, and is therefore fully Endo H sensitive. Note the difference in mobility between Tfr^{dMM} and Tfr (Fig. 3 *a*, lanes *4* and *5*). The difference in mobility between Tfr^{dMM} and Tfr is visible before, as well as after Endo H digestion (Fig. 3 *b*, lanes *4* and *5*). Tfr^{dMM} retains its full Endo H sensitivity even after 24 h of reculture in the absence of dMM (Fig. 3 *b*, lane *11*).

Using IEF it is possible to monitor the addition of (negatively charged) sialic acids. When the same set of samples shown in Fig. 3 *a* and 3 *b* was analyzed by one dimensional IEF, no addition of sialic acids was demonstrable after 24 h of reculture in the absence of dMM, as judged from the complete absence of newly appearing, more acidic species of Tfr (Fig. 3 *c*).

Does the Tfr Carry O-linked Glycans?

We obtained evidence in support of the presence of O-linked sugars on the Tfr. First, the IEF pattern of Tfr from control cells before and after neuraminidase (NANAse) digestion is inconsistent with the presence of a single complex-type N-linked glycan (Fig. 3 *d*, lane *2*). An average of five, and a maximum of six or seven sialic acids can be deduced from the complexity of the IEF banding pattern and NANAse digestions. This finding is incompatible with the presence of

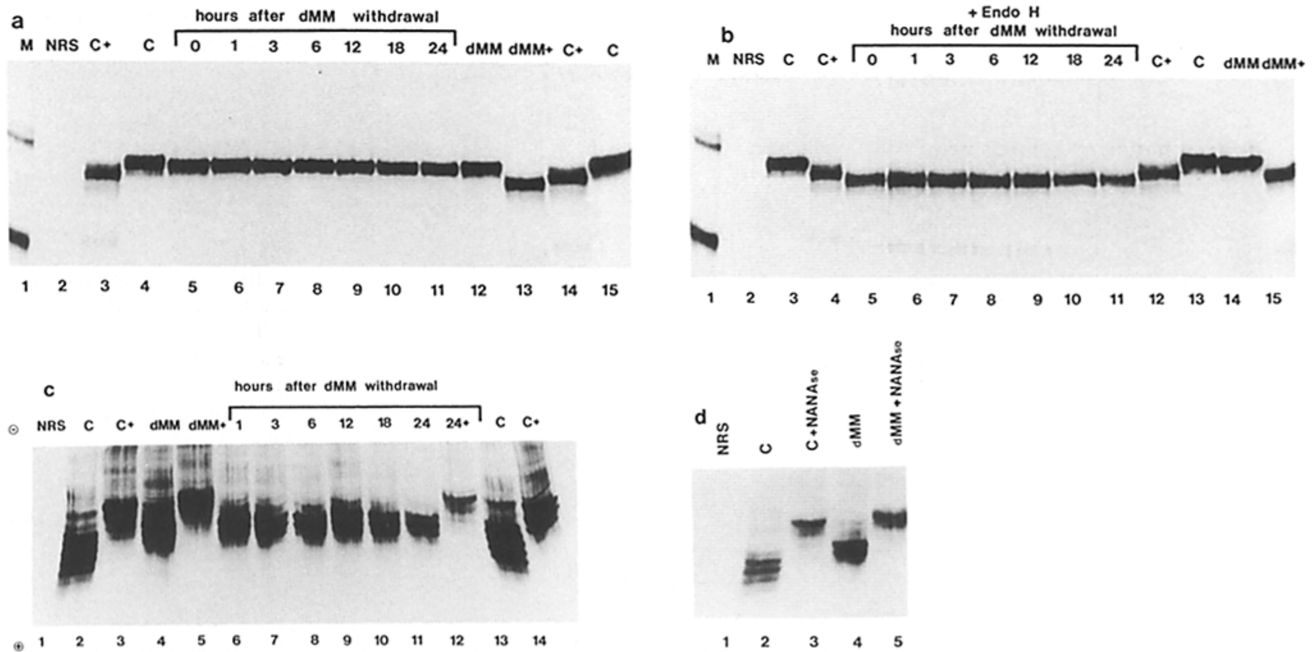

Figure 3. No conversion of Tfr^{dMM} to Tfr upon reculture of dMM-treated K562 cells. Analysis of Tfr immunoprecipitated from K562 lysates after a recycling experiment as described in Materials and Methods. (*a*) Tfr was analyzed from control cells before (*C*, lanes *3* and *14*) and after (*C+*, lanes *4* and *15*) digestion with Endo H. Tfr^{dMM} was analyzed similarly (*dMM*, lane *12*; *dMM+*, lane *13*). Note the clear difference in mobility for dMM + and C+, and the reproducible difference in mobility between C and the 0 timepoints after withdrawal of dMM (lane *5*). After 24 h of dMM withdrawal, there is no noticeable shift in mobility of Tfr^{dMM}. For each sample, equal amounts of radioactivity were loaded. *NRS*, immunoprecipitation with normal rabbit serum; M, marker proteins: 92 and 68 kD, respectively. (*b*) As *a*, but Tfr^{dMM} samples taken after dMM withdrawal were digested with Endo H. Other samples have been labeled as for *a*. Note the difference in mobility between the 24-h withdrawal sample, and the *C+* sample (lanes *11* and *12*). No conversion of Tfr^{dMM} to Tfr has taken place. For each sample, equal amounts of radioactivity were loaded. (*c*) As for *a*, but immunoprecipitated samples were analyzed by one-dimensional IEF. Lanes labeled + were subjected to digestion with NANAse before IEF. Anode is at the bottom. Note the lack of newly appearing sialylated forms of Tfr^{dMM}. The autoradiogram was deliberately overexposed to show that Tfr^{dMM} is not converted to a form with a sialic acid content equivalent to Tfr (compare lanes *11* and *13*). The presence of sialic acids on Tfr^{dMM} suggests that Tfr carries N- and O-linked glycans (see text). A shorter exposure of lanes *1–5* is shown in *d*.

a single tri- or even tetraantennary complex glycan (14, 15, 25). Second, Tfr^{dMM} does not focus at the same position as Tfr or Tfr^{dMM} after NANAse digestion (Fig. 3 *c*, lanes *3* and *5*). Treatment with dMM reduces the average number of sialic acids to three. Note that class I antigens, which carry only a single N-linked glycan and do not contain O-linked sugars (16) are indistinguishable on IEF after NANAse digestion and dMM treatment (Fig. 4). Thus, NANAse sensitivity on glycoproteins labeled in the presence of dMM can occur only if sialic acids are present on O-linked sugars. The position on IEF gels of NANAse-digested, but not that of undigested Tfr^{dMM} is identical to that of NANAse-digested Tfr. Our data are therefore consistent with the presence of O-linked glycans containing an average of two to three sialic acids on Tfr. To our knowledge, the presence of O-linked sugars on Tfr has not been previously suggested. Our observations would explain why the position of migration of Tfr on IEF also differs for NANAse-digested Tfr, and Tfr^{dMM}, in the report of Snider and Rogers (reference 24; Fig. 5).

HepG2

Recycling experiments were also carried out for HepG2 cells, with results essentially similar to those obtained for K562. Tfr as well as class I antigens were precipitated from equal amounts of TCA-precipitable radioactivity. No conversion to control-type Tfr was seen upon reculture of dMM-treated HepG2 cells. No species of Tfr carrying an increased number of sialic acids could be detected upon reculture in the absence of inhibitor of dMM-treated cells, confirming the results obtained for K562 (Fig. 4, *left*).

We also analyzed whether reconversion of high-mannose glycans could take place for another membrane glycoprotein in HepG2 cells, the HLA class I antigens. The single N-linked glycan carried by class I antigens is always of the complex type in control cells (16, 27). Note that dMM treatment results in the generation of class I heavy chains that are indistinguishable by IEF from NANAse-digested control class I molecules (Fig. 4, *right*, lanes *3* and *4*).

As was shown for Tfr, class I molecules did not convert from an Endo H-sensitive to an Endo H-resistant form (results not shown) nor could the addition of sialic acids upon reculture in the absence of dMM be substantiated by IEF (Fig. 4, *right*, lanes *4–9*).

Reconversion of High-Mannose Oligosaccharides on Membrane Proteins in Lymphoid Cells

To establish that the lack of conversion of high-mannose to complex-type sugars on HLA class I molecules was not re-

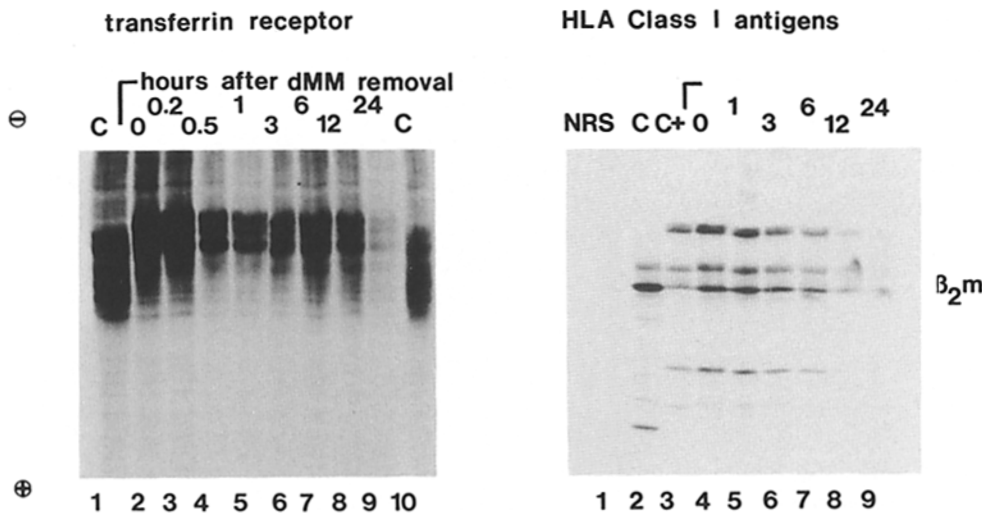

Figure 4. Reculture of dMM-treated HepG2 cells does not result in reconversion of high-mannose N-linked glycans. A recycling experiment was carried out on HepG2 cells as described in Materials and Methods, similar to the experiment described in Fig. 3. Immunoprecipitations were carried out from constant amounts of input radioactivity. The decrease in amount of immunoprecipitated material thus in both panels reflects turnover of surface-labeled proteins. (*Left*) Analysis of Tfr by one-dimensional IEF. No acquisition of sialic acids is detectable for Tfr^{dMM} even after 24 h of reculture. (*Right*) Analysis of HLA class I antigens by one-dimensional IEF. No acquisition of sialic acid is detectable for class I heavy chains. Note that the pattern observed for dMM-treated material is identical to that observed for NANAse-digested control samples (*C+*).

stricted to the HepG2 cell line, a similar experiment was carried out for the human lymphoblastoid cell line JY. Class I antigens have been reported to undergo recycling in lymphoid cells (18). For class I antigens in the JY cell line, no reconversion of high-mannose to complex-type oligosaccharides could be detected by acquisition of Endo H resistance (Fig. 5 *a*) upon reculture of dMM-treated JY cells in the absence of inhibitor.

A similar analysis was performed for HLA class II antigens. These molecules consist of an α chain, which normally caries a high-mannose and a complex-type oligosaccharide, and a β chain, which carries a single complex-type glycan (21). In experiments similar to those described above for class I antigens, no acquisition of sialic acids could be documented even after 24 h of reculture of dMM-treated cells in the absence of dMM (Fig. 5 *b*).

Recycling of Bulk N-linked Glycoproteins along the ER/cis-Golgi

We have been unable to demonstrate acquisition of complex-type sugars upon reculture of dMM-treated cells in the absence of inhibitors. These findings extend to the Tfr for two different cell lines, HLA class I molecules for two cell lines, and HLA class II molecules for one cell line. These findings cannot exclude that certain other glycoproteins do recycle through the *cis*-Golgi. To address this problem, we labeled K562 cells with 2-[^{3}H]-D-mannose in the presence and absence of dMM for 45 min, and chased for 1 h. Cells were recultured in the presence or absence of 20 mM mannose, to be able to observe the possible reutilization of labeled mannose derived from glycoprotein catabolism. Samples were taken at 0 and 18 h after removal of dMM. Lysates were prepared and digested with pronase. Samples were analyzed through a Mono Q column as described (28), resulting in the separation of charged (complex) sialylated and noncharged (high-mannose) carbohydrates. 2-[^{3}H]-D-mannose cannot be subjected to epimerization into other sugars without losing its label and mannose is only incorporated into N-linked carbohydrates. Fig. 6 shows the result of the analysis of these samples. A difference between the carbohydrate pattern from control and dMM-treated cells is apparent, the fraction of complex carbohydrates (fractions 16–45) found in dMM-treated cells most probably being due to glycoproteins that escaped inhibition by dMM. However, after 18 h of reculture of dMM-treated cells, no significant increase of complex-type carbohydrates is observed (compare *right-sided graphs* in Fig. 6), as is obviously also the case for the carbohydrate chain from control cells (Fig. 6, *left-sided graphs*). Cells recultured in the presence of excess mannose showed radioactivity recovery reduced by 5 (labeled control cells) or 9% (cells labeled in the presence of dMM), indicating that some reutilization of label can take place. The distribution of label over the chromatographic profile was not altered by the inclusion of excess mannose in the medium during reculture. Thus for total glycoproteins in K562 cells, no conversion of high-mannose oligosaccharides to complex-type glycans is observed. Significant recycling along the *cis*-Golgi is therefore unlikely.

Discussion

The routes traveled by recycling receptors have been particularly well defined, both biochemically and ultrastructurally as far as the endocytic portion of the pathway is concerned. Far less is known about the exocytic route(s) followed by recycling receptors.

One report describes the addition of sialic acid to surface-labeled, neuraminidase-digested surface molecules, the Tfr (22). This finding was interpreted as evidence that recycling receptors pass through the compartment where sialyltransferase is located, the *trans*-Golgi network. The kinetics of resialylation, when compared with the kinetics of recycling (22), indicate that only a fraction of recycling receptors is a substrate for sialyltransferases on a per cycle basis.

Two recent reports claim that the *cis*-Golgi, the site of action of mannosidase I, is also an integral part of the route taken by surface-derived recycling receptors. In one study (32), electron micrographical evidence supported the notion

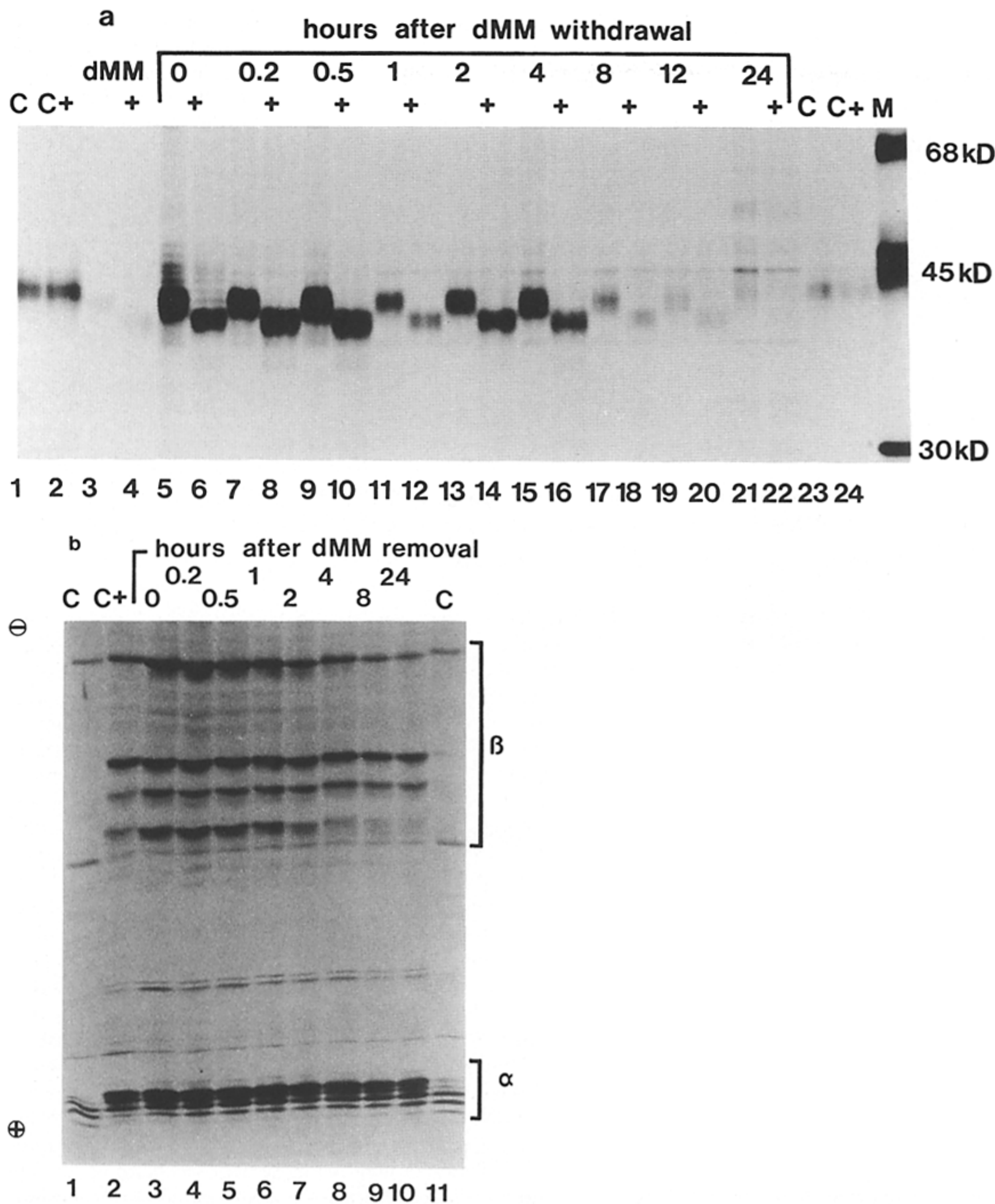

Figure 5. Glycans of HLA antigens from dMM-treated JY cells retain the high-mannose configuration upon reculture. A recycling experiment similar to those shown in Figs. 3 and 4 was carried out on JY cells. Immunoprecipitations were carried out from constant amounts of input radioactivity. (*A*) Analysis of HLA class I antigens by SDS-PAGE. Lanes indicated + represent Endo H-digested samples. Otherwise the organization of the figure is as for Fig. 3. The disappearance of the class I heavy chain with time reflects its turnover in the course of the experiment. (*B*) Analysis by IEF of class II antigens. Position of migration of the α and β chains is indicated by brackets. Note that the NANAse-digested samples show a pattern comparable to dMM-treated class II antigens. Although there appears to be selective decay of some polypeptides, no newly sialylated species arise even after 24 h.

that Tfr, tagged at the cell surface with anti-Tfr antibodies or fragments thereof, traveled to the *cis*-Golgi within 10 min. After 30 min, distribution of surface-tagged Tfr over the different Golgi compartments was random. This suggested selective transport of recycling receptors, first through the *cis*-Golgi, followed by transport through the *trans*-Golgi network. The second study used techniques similar to those used in the present report (24). Surface Tfr, synthesized in the presence of dMM, were analyzed with respect to their type of N-linked glycan after reculture of cells in the absence of dMM. Any reconversion of high-mannose glycans (being the result of inhibition by dMM) to complex-type N-linked glycans analyzed by acquisition of Endo H resistance or of sialic acid heterogeneity would be indicative of passage

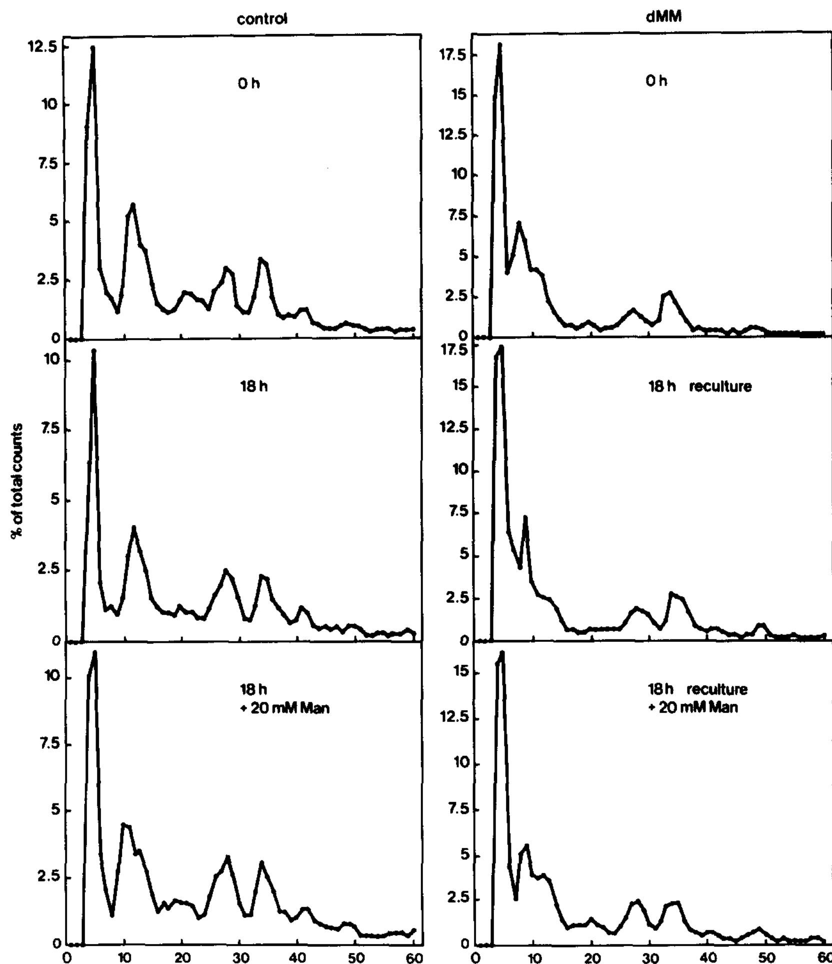

Figure 6. Analysis of total N-linked glycans of K562 cells. K562 cells were labeled with 2-[³H]mannose in the absence (*left side*) and the presence of dMM (*right side*). Total cell lysates were digested with pronase and analyzed by FPLC on a Mono Q column. Radioactivity in each fraction was determined by liquid spectrometry. The radioactivity in each fraction was then calculated as the percentage of total number of counts per minute recovered for the entire elution profile. (*Top*) Total pattern after 0 h. (*Center*) Total pattern after 18-h reculture. (*Bottom*) Total pattern after 18-h reculture in the presence of 20 mM mannose. Noncharged species are eluted first from the column (fractions 1-10, as deduced from NANAse treatment of sialylated oligosaccharides). Fractions 16-24, 24-31, 31-40, and 40-45 are the one sialic acid, two sialic acid, three sialic acid, and four sialic acid-containing species, respectively, as determined through FPLC analysis of glycopeptides derived from α_1-acid glycoprotein before and after NANAse digestion. Abscissa, fraction number; ordinate, percent of total counts.

along the site of mannosidase I, the endoplasmic reticulum/*cis*-Golgi up to the site of action of GlcNAc transferase I and mannosidase I, the medial Golgi. Apart from the fact that both studies (24, 31) report widely different kinetics for the delivery of recycling glycoproteins to the *cis*-medial vs. the *trans*-Golgi, possibly due to the different experimental protocols used, there are several problems of interpretation with the latter study. First, because the inhibitory effect of dMM is completely reversible (24; this study, Fig. 1), Tfr produced upon reculture of cells labeled in the presence of dMM should be indistinguishable from control Tfr if recycling through the *cis*-Golgi would indeed occur. This is obviously not the case even after 20 h of reculture (reference 24; Figs. 3-5). Second, the analysis of bulk [³H]mannose-labeled glycopeptides in dMM-treated cells before and after reculture in the absence of dMM suffers from the fact that after 18 h of reculture, only 20% of the label present at 0 h of reculture was actually recovered in the study of Snider and Rogers (24). There is no net increase in radioactivity in complex oligosaccharide-containing fractions. Thus, the argument in support of reconversion of high-mannose to complex-type oligosaccharides is indirect, because it rests on the assumption that there is no selective breakdown of high-mannose oligosaccharide-containing structures. It is remarkable that the relative loss of label in [³H]mannose-labeled Tfr is substantially less during reculture than that observed for bulk glycopeptides (compare Figs. 3 and 6 in reference 24). Third, even upon reculture of [³H]mannose-labeled cells that had never been exposed to dMM, following the same experimental protocols, the relative increase in complex-type sugars over the 18-h reculture period was of the same order of magnitude as that observed for the reculture of dMM-treated cells (compare Figs. 4 and 7 in reference 24). Because the former figures must necessarily represent completion of high-mannose oligosaccharide trimming and terminal carbohydrate processing of newly synthesized labeled molecules, corrections should be applied to values observed for reculture of dMM-treated cells. The increase in complex-type oligosaccharides observed for control cells (Fig. 7 in reference 24) represents the experimental error of the system, and, as stated, this error is of the same order of magnitude as the experimentally observed values. Finally, even though Tfr has a recycling time which is amongst the shortest reported (5 min [30]), the rate of reconversion of Tfr high-mannose glycans, upon reculture of dMM-treated cells, is lower than that of bulk oligosaccharides (more than 6 and 4 h, respectively; Figs. 3 and 6 of reference 24).

We were able to confirm (24), for all three cell lines tested, the complete reversibility of inhibition by dMM. We also confirmed that cells, grown in the presence of dMM for periods sufficiently long to produce Tfr containing exclusively high-mannose N-linked oligosaccharides on the cell

surface, were indistinguishable from control cells as far as Tfr binding and recycling of Tfr are concerned. Surface expression of Tfr, as measured by immunoprecipitation of surface-iodinated material, was quantitatively comparable for control and dMM-treated cells.

Our observations that Tfr^{dMM} is clearly distinct from Tfr upon IEF deserves comment. We suggest that O-linked glycosylation of Tfr takes place, and that this modification has been overlooked thus far. The difference in IEF banding pattern observed between dMM-treated and control Tfr can be abolished by NANAse treatment. Our results do not support the conclusions of Snider and Rogers (24) as far as return of recycling glycoproteins to the site of action of mannosidase I is concerned. We have been unable to detect any reconversion of high-mannose oligosaccharides on Tfr from dMM-treated cells after periods of reculture as long as 24 h as found earlier, surprisingly also by Snider and Rogers (23). Our observations were made in three dissimilar types of cells: the human erythroleukemic cell line K562, the same cell line as was used by Snider and Rogers; the human lymphoblastoid cell line JY; and the human hepatoma cell line HepG2. No reconversion could be detected either for class I histocompatibility antigens in the lymphoblastoid and hepatoma cell lines, or for class II histocompatibility antigens in the lymphoblastoid cell line. We could confirm and extend our earlier experiments of this type on lymphoid cells that were restricted to shorter periods of observation (12 h) (20). We therefore disagree with the notion (24) that all glycoproteins return to the site of action of mannosidase I with half-times of 4–6 h. In fact, one cannot fail to be impressed with the precision with which the cell keeps these potentially intersecting pathways separate, as judged from the biochemical evidence presented here. Even though small peptides, when added to intact cells, can be transported back as far as the ER (31) where they then enter the secretory pathway, the experiments reported here show that for Tfr, HLA class I and II antigens as well as bulk glycoproteins, the *cis*/medial Golgi is unlikely to be revisited for the better part of their lifetime.

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