Evidence for Recycling of the Resident medial/trans Golgi Enzyme, N-Acetylgalactosaminyltransferase I, in ldlD Cells*

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IdlD cells, which lack the UDP-Gal/UDP-GalNAc 4-epimerase, were stably transfected with a Myc-tagged version of N-acetylgalactosaminyltransferase I (Myc-GlcNAc-T I). In the absence of GalNAc and Gal, newly synthesized GlcNAc-T I did not acquire O-linked oligosaccharides but was catalytically active and was transported to the Golgi region as defined using both immunofluorescence and immunoelectron microscopy. After addition of cycloheximide to prevent further synthesis, GalNAc and Gal were added, and the unglycosylated GlcNAc-T I was found to acquire mature, O-linked sugar, GalNAc, is added followed by anterograde transport back to the Golgi stack, where addition of Gal and sialic acid occurs.

Transport from the endoplasmic reticulum (ER)1 to the cell surface occurs by default. No signals are needed for proteins to move from one compartment to the next along the exocytic pathway (Pfeffer and Rothman, 1987; Pryer et al., 1992; Rothman and Orci, 1992). Signals are needed to direct proteins in the trans Golgi network (TGN) to organelles such as the lysosomes (Kornfeld and Mellman, 1989) and secretory granules (Bauerfeind and Huttner, 1993). They are also needed to localize proteins to particular compartments on the exocytic pathway. These signals are of two types: retention signals and retrieval signals (Nilsson and Warren, 1994). Retention signals provide the primary means of localizing a protein to a particular compartment. The best characterized are those located in the membrane-spanning domain (and flanking sequences) of Golgi enzymes, though the precise manner in which they work has still to be elucidated (Bretscher and Munro, 1993; Nilsson et al., 1993b). Retrieval signals have been identified on soluble proteins in the ER (Munro and Pelham, 1987) and on membrane proteins both in the ER (Nilsson et al., 1988a; Jackson et al., 1993; Schutze et al., 1994) and the TGN (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). These function to return proteins to their correct compartments after they have left them. The act of leaving may reflect the limitations of the retention mechanism in which case retrieval is a salvage process (Warren, 1987). Alternatively, it may reflect the transport process, which requires reusable proteins to package and deliver cargo to the next compartment. In this case, retrieval is a recycling process (Pelham, 1989).

In one case, that of TGN38 (Ponnambalam et al., 1994), a retention signal has been identified in the membrane-spanning domain and a retrieval signal in the cytoplasmic tail. This suggests that all proteins along the exocytic pathway might be equipped in this manner, with the membrane-spanning domain providing the primary means of localizing the protein and the retrieval signal acting to return the protein should it either deliberately or accidentally leave the compartment in which it mostly resides.

Retrieval signals have not yet been identified in resident Golgi enzymes, though there is some evidence that they are retrieved from later compartment(s). Mannosidase II, for example, is present in medial/trans cisternae in most cells (Velasco et al., 1993), yet the bound oligosaccharides are modified by sulfate residues (Moremen and Touster, 1985), a modification that is restricted to the TGN (Bauerfeind and Huttner, 1987). Other Golgi proteins have also been shown to contain oligosaccharides that suggest passage beyond the point at which they normally reside (Yuan et al., 1987; Gonatas et al., 1989; Jinshon et al., 1994; Alcalde et al., 1994). It is, however, possible that these modifications are caused not by recycling but by the presence of small amounts of the oligosaccharide-modifying enzymes in compartments other than those in which they mostly reside. We have shown recently that enzymes involved in the construction of complex N-linked oligosaccharides are mostly present in two adjacent cisternae, but small amounts are present in flanking cisternae (Nilsson et al., 1993a).

We set out to devise a system that would measure the retrieval of a resident Golgi enzyme. We exploited the properties of a Chinese hamster ovary cell line (IdlD) generated by Krieger and colleagues (Kingsley et al., 1986), which lacks the epimerase activity needed to synthesize Gal and GalNAc from glucose precursors. The consequence is that synthesis of O-linked oligosaccharides cannot be initiated, and N-linked oligosaccharides are truncated at the point where terminal Gal would have been added. These defects are rapidly reversed by the addition of exogenous Gal and GalNAc.

The synthesis of O-linked oligosaccharides is initiated in the

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1 The abbreviations used are: ER, endoplasmic reticulum; NEM, N-ethylmaleimide; TGN, trans-Golgi network; CGN, cis-Golgi network; GalNAc-T, galactosaminyl transferase Myc-GlcNAc-T I, Myc-tagged glucosaminyl transferase I; GlcNAc-T I, glucosaminyl transferase I; dFCS, dialyzed fetal calf serum; PAGE, polyacrylamide gel electrophoresis.
Recycling of a Golgi Enzyme

CGN (Tooze et al., 1988; Niemann et al., 1982; Krijnse-Locker et al., 1994) and the cis cisterna of the Golgi stack (Roth, 1984; Deschenes et al., 1988; Roth et al., 1994) by the addition of GalNAc to serine or threonine residues. The CGN (Huttner and Tooz, 1989; Hsu et al., 1991; Pelham, 1989) also known as the intermediate (Saraste and Kuismanen, 1984; Schweizer et al., 1990) or salvage compartment (Warren, 1987), receives the intermediate (Saraste and Kuismanen, 1984; Schweizer et al., 1990) or salvage compartment (Warren, 1987), receives the intermediate. Some samples were incubated for 3 h at 37 °C in 50 μl of 25 mM sodium phosphate buffer, pH 6.0, containing 8–10 units of neuraminidase type III (Glyko, Inc.). Proteins were fractionated on 8–10% polyacrylamide gels (Laemmli, 1970) and transferred using the buffer system of Towbin et al. (1979). Myc-GlcNAc-T I was detected using the 9E10 monoclonal antibody (ICRF hybridoma unit) as the primary antibody at a dilution of 1:1000 followed by an horseradish peroxidase-coupled goat anti-mouse antibody (Tago; 1:2000 dilution) and visualized using the ECL Western blotting kit from Amersham. The proteins were scanned using an LKB Ultra-Versa XL densitometer. Molecular weights were derived from standard curves constructed using the low range SDS-PAGE standards from Bio-Rad.

Experimental Confocal Microscopy—Cells were processed for immunofluorescence microscopy as described by Warren et al. (1984). They were double labeled for Myc-GlcNAc-T I and mannansidase II using monoclonal 9E10 antibodies and polyclonal anti-mannansidase II antibodies (Moremen and Touster, 1985), respectively. The secondary antibodies were Texas red-conjugated, horse anti-mouse and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Vector Labs). The stained cells were visualized using an MRC1000 confocal laser microscope (Bio-Rad).

Electron Microscopy and Immunogold Labeling—Cells were fixed at room temperature with 2% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 2 h. Fixed cells were processed for cryoelectron microscopy and immunogold labeling exactly as described by Misteli and Warren (1995). The primary 9E10 antibody was used at a 1:100 dilution in PBS, 0.5% fish skin gelatin; the secondary antibody rabbit anti-mouse antibody coupled to 10 nm of gold (Biocell, Cardiff, UK) was used at a dilution of 1:20. Sections were examined in a Phillips CM10 electron microscope.

Morphometric Analysis—Photographs of Golgi areas were taken at a final magnification of 38,750 or 52,000 times. Cisternae, Golgi stacks, and the Golgi area were defined as in Misteli and Warren (1995).

To determine the labeling density, the sectioned surface area of Golgi stacks, the Golgi area, or the nucleus was determined by a point-hit method (Weibel et al., 1969) using the formula A = (P/d^2)M^2, where A = the sectioned surface area, P = the grid intersections falling over the area, d = the distance between grid lines, and M = the final magnification of the photograph.

The number of gold particles associated with the measured area was counted, and the density was determined by N/A, with N being the number of gold particles. For each sample, at least 15 Golgi areas from cells from two experiments were counted, and the results are presented as the mean ± S.D.

Enzyme Assays—Cells were grown to 70–80% confluency, and the harvested cell pellets were extracted with 1% Triton X-114 essentially as described by Bordier (1981), except that the initial extract was centrifuged at 14,000 rpm for 5 min at 4 °C before phase separation at 37 °C. Aliquots of the detergent phases were assayed in duplicate for p14-galactosyltransferase, GlcNAc-T I, and protein content as described previously (Bretz and Staubli, 1977; Vischer and Hughes, 1981; Slusarewicz et al., 1994).

RESULTS

O-Linked Sugars Bound to Myc-GlcNAc-T I—Since suitable antibodies were not available to the endogenous GlcNAc-T I in IdID cells, they were transfected with the cDNA encoding human GlcNAc-T I (Kumar et al., 1990). This was modified so as to encode the Myc epitope at the C terminus of the protein (Nilsson et al., 1993a), which resides in the Golgi lumen. The Myc epitope is recognized specifically and with high affinity by the 9E10 monoclonal antibody (Evans et al., 1985).

This modified cDNA was subcloned into the pSRu vector, and stable transfectants were selected in the presence of gentamicin (Southern, 1981; Canaan and Berg, 1982). Mixed clones were used in which more than 90% of the cells expressed similar levels of protein as assessed by immunofluorescence microscopy.

To determine the composition of the O-linked oligosaccharides on the Myc-GlcNAc-T I, cells were grown for 3 days in McCoy's medium supplemented with insulin, transferrin, and selenium (ITS medium) in the presence or absence of GalNAc/ Gal or in the presence of FCS. After lysis and fractionation by SDS-PAGE, total membrane proteins were transferred to membranes and were probed with the monoclonal antibody 9E10.

Three forms of the protein were detected. An unglycosylated form of 51 kDa, an immature form of 52 kDa, and a mature form of 55 kDa. The unglycosylated form of 51 kDa was the
The molecular mass of 51 kDa is very similar to that calculated from the sequence of the protein, 50.9 kDa (Kumar et al., 1990). A minor form of 52 kDa was also present. The amount of the minor 52-kDa form increased with time in ITS medium. Since this form migrated almost exactly with that obtained by incubating the cells in ITS containing GalNAc (Fig. 1A, lane 2), it seemed likely that it represented an immature form of the protein (see “Discussion”). Sugars such as GalNAc can sometimes be salvaged during degradation of endogenous proteins (Krieger et al., 1989). When cells were incubated in ITS medium containing both GalNAc and Gal, the molecular mass of Myc-GlcNAc-T I increased to 55 kDa (Fig. 1A, lane 3), the same as that obtained in the presence of dFCS (Fig. 1A, lane 4). These mature forms of Myc-GlcNAc-T I were also sensitive to treatment with neuraminidase (Fig. 1B, lanes 1–4), showing that the O-linked oligosaccharides contained sialic acid. Taken together, these data show that a mixture of GalNAc and Gal is both necessary and sufficient for the construction of the O-linked oligosaccharides found on the mature protein. Furthermore, the difference in molecular mass between the unglycosylated and mature forms (4 kDa) is consistent with the addition of two O-linked oligosaccharides (Kornfeld and Kornfeld, 1980).

Activity of Myc-GlcNAc-T I—Cells were extracted with Triton X-114, and the membrane proteins were enriched by partitioning at 30 °C into the detergent phase (Bordier, 1981). GlcNAc-T I was assayed using ovalbumin as the acceptor. Table I shows that the transfected cells had about 10 times the activity of the parental cell line, irrespective of the growth medium or incubation conditions. This increase was specific to GlcNAc-T I because another resident Golgi enzyme, β1,4-galactosyltransferase, was found to have a small but significantly lower specific activity in the transfected cells (Table I). These data argue strongly that the expressed Myc-GlcNAc-T I is enzymatically active and must, therefore, have folded correctly in IdD cells.

Table I also shows that the specific activity of GlcNAc-T I was unaffected by the addition of GalNAc/Gal to the ITS growth medium. Since this treatment adds O-linked oligosaccharides to pre-existing Myc-GlcNAc-T I (see below and Fig. 4), it is clear that these O-linked oligosaccharides have no significant effect on the catalytic activity of the protein. Similar results were obtained for β1,4-galactosyltransferase.

A small but significant increase in the specific activities of both enzymes in both cell lines when the cells were grown in ITS medium containing dFCS likely reflects the presence of growth factors that increase the growth rate.

Location of Myc-GlcNAc-T I—The location of Myc-GlcNAc-T I in IdD cells was determined using both immunofluorescence and immunoelectron microscopy.

When the stably transfected cells were grown under optimal conditions, in the presence of ITS and dFCS, GlcNAc-T I was localized by immunofluorescence to a compact juxtanuclear reticulum characteristic of the Golgi apparatus in these cells (Fig. 2A). This location was confirmed by double labeling for the resident enzyme, mannosidase II (Louvard et al., 1982; Velasco et al., 1993) (Fig. 2B). Exactly the same pattern was obtained when the cells were grown in the presence of ITS medium alone in the absence (Fig. 2, C and D) or presence (Fig. 2, E and F) of GalNAc/Gal. The recycling experiments described below had to be conducted in the presence of cycloheximide, and it was, therefore, important to show that inhibition of protein synthesis had no effect on the distribution of Myc-GlcNAc-T I. Treatment with cycloheximide alone for 8 h had no effect (data not shown) and neither did a 4-h treatment continued for a further 4 h in the presence of GalNAc/Gal (Fig. 2, G and H). In addition, no Myc-GlcNAc-T I was detected to be secreted into the medium (data not shown) under all conditions.

These results were both confirmed and extended by immunogold microscopy on cryosections. For cells grown in the presence of dFCS alone (Fig. 3A) or in the presence of GalNAc/Gal with (Fig. 3D) or without (Fig. 3C) cycloheximide, the results were indistinguishable. Labeling was restricted to stacked Golgi cisternae, and between two and four adjacent cisternae were labeled. There was no significant labeling over any other structure in the cell. Quantitation showed that the level of labeling was also very similar, the average labeling density over Golgi stacks only varying between 24 and 28 gold particles/μm² (Table II).

The only exception to this distribution was found in transfected cells grown in the absence of any additional components, that is, in the presence of ITS medium alone. In these cells, the Golgi membranes appeared to be less cisternal and more tubulo-reticular (Fig. 3B). Golgi stacks were present (inset to Fig. 3B) in about 30% of the cells. The density of gold particle labeling over the Golgi stack was only just over half that of the other samples (Table II). However, this did not mean that Myc-GlcNAc-T I had been lost from the Golgi apparatus because the density of labeling over the entire Golgi region, including the tubular and vesicular structures closely associated with the stacks, was very similar for all the samples (Table II). The glycosylation state of Golgi proteins may, therefore, affect the morphology of the stack, but it did not change the level or Golgi location of Myc-GlcNAc-T I.

Recycling of Myc-GlcNAc-T I—Cells grown in ITS medium were incubated with cycloheximide for 4 h, sufficient time to chase any newly synthesized Myc-GlcNAc-T I to the Golgi region. Incubation was then continued in the presence of cycloheximide for up to 4 h in the presence or absence of GalNAc/Gal. Samples were fractionated by SDS-PAGE and blotted, and the Myc-GlcNAc-T I was detected using the 9E10 antibody.

Addition of GalNAc/Gal resulted in the disappearance of the unglycosylated form of the protein and the appearance of the immature form. This was followed by the appearance of the
The formation of mature Myc-GlcNAc-T I (Fig. 5, lanes 3 and 4), however, was noted (data not shown). The first is that GlcNAc-T I, lacking sialic acid residues (Fig. 4 B), the levels of unglycosylated and immature forms had acquired sialic acid residues (Fig. 4 A). In the absence of GalNAc/Gal and be rapidly degraded. The half-life of the uncleaved and mature proteins at about 10 h, not too dissimilar from the 10–20 h that characterizes the half-life of other Golgi proteins (Strous and Berger, 1982; Yuan et al., 1987). More importantly, the transfected cells were shown to have ~10 times the activity of GlcNAc-T I when compared to the parental cell line. Catalytic activity is probably the most sensitive measure of correct folding. The specific activity of Myc-GlcNAc-T I was also not affected by the absence of O-linked oligosaccharides, arguing strongly that they play no role in correct assembly or functioning of the protein.

The location of Myc-GlcNAc-T I was assessed by both immunofluorescence and immunoelectron microscopy. Myc-GlcNAc-T I was localized to a compact juxtanuclear reticulum that characterizes the Golgi apparatus in animal cells (Louvart et al., 1982). The protein also colocalized exactly with an endogenous Golgi marker, mannosidase II. At the electron microscopic level, the protein was found exclusively in the Golgi region. There was no significant labeling over the ER or plasma membrane.

Though both forms of Myc-GlcNAc-T I were present in the Golgi region, it was clear that the lack of sugars changed the morphology of the Golgi stack. Stacked cisternae were only present in around 30% of the cells, but in all the rest of the cells, the Golgi was a more tubulo-reticular structure, suggesting that the oligosaccharides bound to resident proteins might play a structural role in the organization of the Golgi stack. The simplest idea is to suggest that they contribute toward a luminal Golgi matrix that serves to keep the central portions of cisternae apart. In their absence, apposing cisternal membranes would touch, thereby triggering periplasmic fusion and the formation of a tubulo-reticular structure. These ideas are more fully explored in a recent review (Rothman and Warren, 1994). For the present purposes, it is sufficient that the Myc-GlcNAc-T I is restricted to the Golgi complex region and physically separated from the GalNAc-T that initiates synthesis of O-linked oligosaccharides.

The physical separation of Myc-GlcNAc-T I and GalNAc-T is the second assumption implicit in these experiments. Most work places GlcNAc-T I in the medial/trans cisternae and GalNAc-T in the CGN, though some recent data show that Gal-
NAc-T can be present in the cis cisterna in some cells (Roth et al., 1994). Golgi enzymes are mostly found in two adjacent cisternae, but small amounts are found in flanking cisternae. If this is true for GlcNAc-T, then the results could be explained by the presence of small amounts of this enzyme in medial/trans cisternae. Addition of GalNAc/Gal could have led to slow maturation of Myc-GlcNAc-T I without the need for it to recycle via the CGN.

To demonstrate that this was not occurring and that the maturation of Myc-GlcNAc-T I required vesicular transport, we investigated the effects of several transport inhibitors. Both anterograde (Rothman, 1994) and retrograde (Letourner et al., 1994) transport through the Golgi stack is mediated by COP I-coated vesicles, which bud from one cisterna and fuse with the next on the pathway. Fusion of these vesicles with their target membrane can be inhibited by pretreatment with NEM, which inactivates the general fusion protein, NSF (NEM-sensitive factor) (Glick and Rothman, 1987). Fusion is also inhibited during mitosis (Misteli and Warren, 1994) and leads to the fragmentation of the Golgi apparatus (Warren, 1993). Fragmentation of the Golgi apparatus can be mimicked by the addition of okadaic acid (Lucocq et al., 1991).

Both NEM and okadaic acid completely prevented the maturation of Myc-GlcNAc-T I. It is, of course, possible that these drugs affected the enzymes (and sugar transporters) involved in the oligosaccharide maturation process, but this seems unlikely given the differences in their chemical structure and mode of action. NEM acts on thiol residues, whereas okadaic acid specifically (and non-covalently) inhibits protein phosphatases 1 and 2A (Cohen et al., 1990). Furthermore, NEM has been shown to have no effect on the processing of both N- and O-linked oligosaccharides bound to either proteins (Glick and Rothman, 1987; Rothman, 1987) or glycolipids (Wattenberg, 1990). Okadaic acid has been shown to have no effect on the addition of dolichol oligosaccharides to newly synthesized proteins in the ER (Lucocq et al., 1991). Taken together, these data suggest that the observed addition of O-linked oligosaccharides to Myc-GlcNAc-T I is the consequence of recycling via the CGN.

The half-time for recycling of Myc-GlcNAc-T I as measured by the acquisition of O-linked oligosaccharides was about 150 min. Newly synthesized Myc-GlcNAc-T I matured with a half-
of GalNAcGal yielded two forms of Myc-GlcNAc-T I.

In summary, we have shown that unglycosylated Myc-GlcNAc-T I resides in the Golgi in IdI D cells starved of GalNAc/Gal. Upon addition of these sugars, the protein acquires O-linked oligosaccharides with a half-time of several hours. Acquisition of the sugars is prevented by inhibitors of vesicular traffic, suggesting that the protein is transported first to the CGN and then back to the Golgi stack. Further work is still needed to confirm a role for vesicle-mediated transport, but this system should provide a convenient means of studying recycling pathways within the Golgi apparatus.

Acknowledgments—IdID cells were kindly provided by Monty Krieger, the cDNA-encoding GlcNAc-T I by Tommy Nilsson, the polyclonal anti-mannosidase II antibodies by Kelley Moremen, and the 9E10 monoclonal antibody by the Hybridoma Development Unit at ICRF. We thank Drs. R. C. Hunt, G. Schiavo, G. Stenbeck, and M. Craighead for critical reading of the manuscript and helpful comments.

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J. Biol. Chem. 1995, 270:25057-25063.
doi: 10.1074/jbc.270.42.25057

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