Selective Reentry of Recycling Cell Surface Glycoproteins to the Biosynthetic Pathway in Human Hepatocarcinoma HepG2 Cells

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Abstract. Return of cell surface glycoproteins to compartments of the secretory pathway has been examined in HepG2 cells comparing return to the *trans*-Golgi network (TGN), the *trans/medial-* and *cis*-Golgi. Transport to these sites was studied by example of the transferrin receptor (TfR) and the serine peptidase dipeptidylpeptidase IV (DPPIV) after labeling these proteins with the *N*-hydroxysulfosuccinimide ester of biotin on the cell surface. This experimental design allowed to distinguish between glycoproteins that return to these biosynthetic compartments from the cell surface and newly synthesized glycoproteins that pass these compartments during biosynthesis en route to the surface. Reentry to the TGN was measured in that surface glycoproteins were desialylated with neuraminidase and were monitored for resialylation during recycling. Return to the *trans*-Golgi was traced measuring the transfer of [³H]fucose residues to recycling surface proteins by fucosyltransferases. To study return to the *cis*-Golgi,

surface proteins were metabolically labeled in the presence of the mannosidase I inhibitor deoxymannojirimycin (dMM). As a result surface proteins retained *N*-glycans of the oligomannosidic type. Return to the site of mannosidase I in the *medial/cis*-Golgi was measured monitoring conversion of these glycans to those of the complex type after washout of dMM. Our data demonstrate that DPPIV does return from the cell surface not only to the TGN, but also to the *trans*-Golgi thus linking the endocytic to the secretory pathway. In contrast, no reentry to sites of mannosidase I could be detected indicating that the early secretory pathway is not or is only at insignificant rates accessible to recycling DPPIV. In contrast to DPPIV, TfR was very efficiently sorted from endosomes to the cell surface and did not return to the TGN or to other biosynthetic compartments in detectable amounts, indicating that individual surface proteins are subject to different sorting mechanisms or sorting efficiencies during recycling.

D URING internalization and recycling plasma membrane glycoproteins of different function may return from the cell surface to the *trans*-Golgi network (TGN)[1] thus linking the endocytic to the secretory pathway. Return from the surface to the TGN was clearly demonstrated for receptors, ectoenzymes, the synaptic vesicle marker protein synaptophysin and other not yet characterized proteins that may efficiently recycle through the TGN 5–10 times during their lifespan (Snider and Rogers, 1985; Woods et al., 1986; Fishman and Fine, 1987; Hedman et al., 1987; Duncan and Kornfeld, 1988; Kreisel et al., 1988; Reichner et al., 1988; Stoorvogel et al., 1988;

van Deurs et al., 1988; Brändli and Simons, 1989; Jin et al., 1989; Green and Kelly, 1990, 1992; Prydz et al., 1990). Routes of recycling to the TGN have been suggested to exit from late endosomes and to function as a salvage pathway removing internalized membrane proteins from sequestration to lysosomes (Green and Kelly, 1992). In contrast, conflicting results have been presented for the return of surface proteins to more proximal sites of the biosynthetic route. Whereas recycling to galactosyltransferases in the *trans*-Golgi was shown for the mannose 6-phosphate/insulin-like growth factor-II receptor in the mutant CHO cell line ldlD (Huang and Snider, 1993), return to these sites could not be detected for both the cation-dependent and the cation-independent mannose 6-phosphate receptor in BW 5147 mouse lymphoma cells (Duncan and Kornfeld, 1988). Furthermore, return of surface proteins to fucosyltransferases presumably localized in *trans/medial*-Golgi compartments (for review see Roth, 1987) was shown for the serine peptidase DPPIV in rat hepatocytes (Kreisel et al., 1988), but was so far not confirmed for other proteins. Morphological studies on the recycling itinerary of plasma membrane TfR tagged with

1. *Abbreviations used in this paper.* dMM, 1-deoxymannojirimycin; DPPIV, dipeptidylpeptidase IV; Endo H, endo-β-*N*-acetylglucosaminidase H; GSH, glutathione; NHS-SS-biotin, sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate; PNGase F, peptide N⁴-(*N*-acetyl-β-glucosaminyl) asparagine amidase F; TfR, transferrin receptor; Tf, transferrin.

anti-TfR antibodies in mouse myeloma cells indicated that there is considerable membrane traffic from the cell surface even to middle and cis-Golgi cisternae (Woods et al., 1986). This assumption was supported by the finding that surface TfR may return to mannosidase I in cis-Golgi compartments of erythroleukemia K562 cells (Snider and Rogers, 1986). On the other hand, Neefjes et al. (1988) were unable to confirm that the TfR or HLA class II antigens return to mannosidase I in the cis-Golgi in different cell lines. Moreover, studies on the cation-dependent and -independent mannose 6-phosphate receptor showed that neither one of these two receptors does routinely return to cisternae containing mannosidase I (Duncan and Kornfeld, 1988). Hence, it remains unknown if cell surface proteins may traffic back as far as to the trans-Golgi and to medial/cis-Golgi compartments. The conflicting results on the return of surface glycoproteins to trans-, medial-, and cis-Golgi compartments may be due to the fact that recycling was in part studied by means of proteins that had not been labeled on the cell surface, or had been labeled noncovalently with ligands or antibodies that may dissociate from the labeled proteins during endocytosis and recycling. Hence, proteins recycling from the cell surface to biosynthetic compartments could not unequivocally be distinguished from newly synthesized proteins en route to the cell surface. As it has to be assumed that the majority of glycoproteins trafficking through the Golgi apparatus represents newly synthesized glycoproteins, whereas cell surface glycoproteins returning to the Golgi apparatus or to the TGN make up only a minor fraction (Reichner et al., 1988; Brändli and Simons, 1989), studies on this route of membrane protein traffic connecting the endocytic to the biosynthetic route crucially depend on the reliable differentiation of recycling cell surface glycoproteins from newly synthesized ones.

Therefore, in the present study reentry of recycling surface proteins to the biosynthetic pathway was examined in HepG2 cells using a novel experimental approach that allows to unambiguously discriminate surface glycoproteins recycling from the cell surface to compartments of the biosynthetic pathway from newly synthesized glycoproteins trafficking through biosynthetic compartments en route to the cell surface. The novel experimental approach uses two criteria for differentiation, (a) covalent labeling of cell surface glycoproteins with NHS-SS-biotin and (b) generation of differences in the glycan moiety of glycoproteins recycling from the cell surface as compared to the glycan moiety of newly synthesized glycoproteins en route to the cell surface. The use of two criteria provided very high powers of differentiation and allowed to trace return of surface glycoproteins to the biosynthetic pathway under utmost reliable conditions. Employing this technique return of proteins from the surface was systematically examined comparing return to peripheral, medial and more proximal sites of the biosynthetic route, i.e., to the TGN, to trans/medial-Golgi cisternae as well as to the cis-Golgi. Reentry of recycling surface glycoproteins to these sites was traced using oligosaccharide modifications effected on recycling glycoproteins by processing glycosidases and glycosyltransferases subcompartmentalized within the biosynthetic pathway as markers (for review see Roth, 1987; Paulson and Colley, 1989). Two surface glycoproteins with

different internalization characteristics were compared: the human TfR that is efficiently internalized with a short half-life of ~5 min at the cell surface (Ciechanover et al., 1983), and the serine peptidase DPPIV (CD26) that is internalized at a comparably low rate (Matter et al., 1990). Neither one of the two proteins returned to the sites of mannosidase I in detectable amounts and, hence, do not significantly recycle to cis-Golgi cisternae. In contrast, DPPIV, but not TfR was recycled from the surface back to the trans-Golgi as well as to the TGN. These results demonstrate that cell surface proteins in HepG2 cells may not only return to the TGN, but also to the trans-Golgi, whereas earlier parts of the secretory pathway are not or are only at insignificant rates accessible to recycling surface proteins.

Materials and Methods

Materials

Constituents of cell culture media were obtained from Gibco (Eggenstein, Germany); other materials for cell culture were purchased from Falcon (Heidelberg, Germany) or Nunc (Wiesbaden, Germany). L-[^{35}S]Methionine (>48 TBq/mmol), L-[6-^{3}H]fucose (>800 GBq/mmol), D-[6-^{3}H]galactose (0.74 TBq/mmol), D-[2-^{3}H]mannose (370 GBq/mmol), [^{125}I] sodium iodide (3.7 GBq/ml) and UDP-D-[U-^{14}C]galactose (7.4 GBq/mmol) were from Amersham Buchler (Braunschweig, Germany). BSA, NP-40, streptavidin agarose, deferoxamine mesylate, DL-dithiothreitol, and iodoacetamide were purchased from Sigma (Deisenhofen, Germany). Sulfosuccinimidyl-2-(biotiamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin), sulfosuccinimidyl-6-(biotinamido)hexanoate, and IodoGen iodination reagent (1,3,4,6-tetrachloro 3α-6α-diphenylglycoluril) were obtained from Pierce (Oud Beijerland, The Netherlands). Protein A–Sepharose and ampholines (pH 3.5–9.5, 40%) were from Pharmacia (Freiburg, Germany). Endo-β-N-acetylglucosaminidase H (Endo H) from Streptomyes plicatus (EC 3.2.1.96), peptide N^4-(N-acetyl-β-glucosaminyl) asparagine amidase F (PNGase F) from Flavobacterium meningosepticum (EC 3.2.2.18), neuraminidase from Vibrio cholerae (EC 3.2.1.18), and dMM were from Boehringer Mannheim (Mannheim, Germany). Neuraminidase from Clostridium perfringens was from Sigma. Oligomannosidic oligosaccharides Man$_{5-9}$GlcNAc$_2$ used as standards were from Oxford Glycosystems (Abingdon, UK). Unless otherwise stated, all other chemicals and reagents were of analytical grade and were obtained either from Sigma or from Serva (Heidelberg, Germany).

Animals, Cells, and Antibodies

Male Wistar rats (120–150 g) were fed a commercial diet (Altromin; Altromin GmbH, Lage, Lippe, Germany) and water ad lib. HepG2 cells (Knowles et al., 1980) (obtained from the American Type Culture Collection [ATCC], Rockville, MD) were grown in DME (Gibco), supplemented with 10% (by vol) heat-inactivated FCS (Gibco), insulin (0.08 mU/ml), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) (complete DME) in a humidified atmosphere with 5% CO$_2$ at 37°C. Cells (2.5 × 10^6) were seeded into 100-mm tissue culture dishes and were used 24 h later at 70% confluence. OKT9 hybridoma cells (Sutherland et al., 1981) (obtained from the ATCC) producing mAb reactive with human TfR were grown in low protein hybridoma medium (Gibco) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 2 mM L-glutamine. mAb OKT9 was purified from hybridoma cell culture supernatants by affinity chromatography on protein A–Sepharose. Ascites fluid containing mAb HBB3/775 recognizing human DPPIV was produced in pristane-primed BALB/c mice (Hauri et al., 1985). Affinity-purified rabbit anti–mouse IgG was from Dakopatts (Hamburg, Germany). The antibodies used in this study are directed against protein epitopes and do not recognize carbohydrate antigens on the glycoproteins.

Metabolic Labeling of Cells

For labeling with L-[^{35}S]methionine, cells were washed and preincubated with MEM with Earle's salt without L-methionine and L-cysteine for 30

min. Cells were then grown for 4 h in MEM without L-methionine/L-cysteine containing L-[^{35}S]methionine (5.5 MBq/3 × 10^6 cells), washed, and chased for 2 h in complete DME containing 1 mM unlabeled L-methionine. For labeling with radioactive sugars, cells were cultured for 6 h in complete DME containing either L-[6-^{3}H]fucose (15 MBq/3 × 10^6 cells), D-[2-^{3}H]mannose (18 MBq/3 × 10^6 cells), or D-[6-^{3}H]galactose (7.4 MBq/3 × 10^6 cells). Cells were then washed and chased for 2 h in medium supplemented with 1 mM unlabeled sugar.

Cell Surface Biotinylation

Cell surface proteins were labeled with biotin essentially as described (Busch et al., 1989). After cooling on ice, cells were washed four times with ice-cold PBS/Ca^{2+}/Mg^{2+} (8 mM Na$_2$HPO$_4$, 1 mM KH$_2$PO$_4$, pH 7.2, 3 mM KCl, 137 mM NaCl, 0.9 mM CaCl$_2$, 0.5 mM MgCl$_2$) and incubated with a freshly prepared solution (1 mg/ml) of NHS-SS-biotin in PBS/Ca^{2+}/Mg^{2+} for 20 min on a rocking platform at 4°C. Cells were then washed twice with PBS/Ca^{2+}/Mg^{2+} containing 0.1% (mass/vol) BSA, twice with PBS/Ca^{2+}/Mg^{2+}, and were then either recultured under the conditions described above or harvested for further analysis.

Preparation of Cellular Membranes from HepG2 Cells

A membrane fraction of HepG2 cells was prepared by the method of Hortsch (1994) with some modifications. Cells were washed, scraped from the dishes with a rubber policeman, and collected in homogenization buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA; 0.25 ml/3 × 10^6 cells), containing 1 mM PMSF, and the protease inhibitors pepstatin, leupeptin, antipain, and chymostatin at a final concentration of 10 µg/ml each. Cell suspensions were homogenized by 15 strokes in a tight-fitting Dounce glass homogenizer on ice and centrifuged (5 min, 500 g). Supernatants were collected and kept on ice. The pellets were once more extracted in homogenization buffer by 15 strokes in a Dounce homogenizer followed by centrifugation (5 min, 500 g). Both supernatants were pooled and cleared by a third centrifugation step (5 min, 2,500 g). The finally obtained pellet was discarded, and membranes were obtained from the supernatants by ultracentrifugation. For this, centrifugation tubes were filled with 0.6 ml of homogenization buffer containing 0.25 M sucrose, overlaid with 3.75 ml of supernatant and centrifuged (60 min, 100,000 g). The pelleted membrane fraction was resuspended in Golgi buffer (100 mM NaAc, pH 6.0, 0.1% NP-40 [by vol]) by 15 strokes in a Dounce homogenizer.

Preparation of Golgi Membranes from Rat Liver

Golgi apparatus was isolated from rat liver using published procedures (Tauber et al., 1986). Purity of the isolated Golgi fraction was checked by electron microscopy and by assay of the marker enzymes glucose-6-phosphatase, galactosyl transferase, 5'-nucleotidase and succinate-INT-reductase with methods given in the references (DeDuve and Appelmans, 1955; Pennington, 1961; Mitchell and Howthorne, 1965; Bauer et al., 1974).

Immunoaffinity Absorption

All steps were carried out at 4°C. Cells were washed four times with ice-cold PBS/Ca^{2+}/Mg^{2+}, scraped from the dishes with a rubber policeman and collected in lysis buffer A (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% (by vol) NP-40; 0.75 ml/3 × 10^6 cells), containing 1 mM PMSF, and the protease inhibitors pepstatin, leupeptin, antipain, and chymostatin at a final concentration of 10 µg/ml each. For detergent extraction, cell suspensions were homogenized by 10 gentle strokes in a tight-fitting Dounce homogenizer and rotated end-over-end for 2 h. Detergent-insoluble material was removed by centrifugation (100,000 g, 30 min). Protein concentration in the supernatants was determined by the bicinchoninic acid assay (Pierce) using BSA as standard. Supernatants were diluted with lysis buffer B (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% [mass/vol] globulin-free BSA, 1% [by vol] NP-40) to a concentration of cell proteins of 0.2 mg/ml, followed by addition of pepstatin, leupeptin, antipain, and chymostatin to a final concentration of 2 µg/ml each. For immunoaffinity absorption 10 µg of monoclonal anti-TfR antibody OKT9 or 4 µl of ascites fluid containing anti-DPPIV mAb HBB3/775 were added to 5 ml of supernatant and rotated end-over-end for 2 h. TfR/mAb OKT9 immunocomplexes were collected by binding to protein A–Sepharose CL-4B precoated with affinity-purified rabbit anti–mouse IgG. For precoating, protein A–Sepharose (10 mg) was incubated with rabbit anti–mouse IgG (3 mg) in lysis buffer B for 2 h and washed twice in the same buffer.

Precoated protein A–Sepharose beads were added to 5 ml of the mixture, rotated end-over-end for 12 h, pelleted by centrifugation, washed once with washing buffer A (50 mM Tris/HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA) with 1% (by vol) NP-40, 0.1% (mass/vol) BSA, twice with washing buffer A with 1% (by vol) NP-40, once with washing buffer A with 0.1% (by vol) NP-40, once with washing buffer A with 0.1% (by vol) NP-40 and 0.5% (mass/vol) SDS, and once with PBS. Immunocomplexes coupled to protein A–Sepharose were eluted either by heating (95°C) for 3 min with 90 µl SDS electrophoresis sample buffer (2% [mass/vol] SDS, 28.6% [by vol] glycerol, 5% [by vol] mercaptoethanol, 0.01% [mass/vol] bromophenol blue, 62.5 mM Tris/HCl, pH 6.8) (for SDS-PAGE) or by shaking three times for 5 min with each 30 µl of 3 M KSCN, 0.5% (by vol) NP-40 at room temperature (for chromatography on streptavidin agarose).

DPPIV/mAb HBB3/775 immunocomplexes were collected by adsorption to protein A–Sepharose that was preincubated with detergent extracts of nonlabeled cells in order to block unspecific binding sites. For preincubation, protein A–Sepharose (10 mg) was incubated with 50 µl of detergent extract (1 mg of protein/ml lysis buffer A) and 500 µl lysis buffer B for 2 h, and washed twice with lysis buffer B. Incubation of precoated protein A–Sepharose with the immunoadsorption mixture, washing of the beads and elution of immunocomplexes was done as described for TfR, except that SDS was used at a concentration of 0.1% instead of 0.5% in the last washing buffer.

Isolation of Biotinylated Proteins

For isolation of total biotinylated proteins from cell homogenates, streptavidin agarose (100 µl) washed four times in lysis buffer A was added to 1 ml of detergent lysate of cells (1 mg protein/ml lysis buffer A) containing antipain, chymostatin, leupeptin, and pepstatin at a concentration of 20 µg/ml each, and rotated end-over-end at 4°C for 2 h. Streptavidin agarose was then pelleted by centrifugation, washed four times with 50 mM Tris/HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1% (by vol) NP-40, 1 M KSCN, twice with the same buffer without KSCN, and once with PBS. Proteins bound to streptavidin agarose were eluted with 50 mM DL-dithiothreitol in 10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% (by vol) NP-40.

For isolation of biotinylated TfR and DPPIV, both proteins were immunoadsorbed beforehand (see above) and eluted from protein A–Sepharose with 3 M KSCN, 0.5% (by vol) NP-40. Eluates were diluted 1:10 in dilution buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% [by vol] NP-40). Streptavidin agarose (25 µl) washed four times in dilution buffer was added to the eluates obtained from 1 mg protein of detergent extract and diluted with dilution buffer to a final volume of 1 ml. After addition of leupeptin, antipain, pepstatin, and chymostatin to a final concentration of 10 µl/ml each, suspensions were rotated end-over-end at 4°C for 2 h, washed four times in 50 mM Tris/HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1% (by vol) NP-40, 0.3 M KSCN, twice in the same buffer without KSCN, and once with PBS. Proteins bound to streptavidin agarose were eluted with 50 mM DL-dithiothreitol in dilution buffer or with SDS electrophoresis sample buffer.

SDS–Polyacrylamide Gel Electrophoresis

Proteins were separated on 7.5% SDS–polyacrylamide slab gels (Laemmli, 1970) and stained with silver (Heukeshoven and Dernick, 1988). Gels with ^{35}S- or ^{3}H-labeled samples were processed and fluorographed according to Bonner and Laskey (1974) using Kodak XAR-5 x-ray film. Bands on fluorograms were quantitated by densitometry using a Hirschmann Elscript 400 Scanner (Hirschmann, München, Germany). Molecular mass standards were α_2-macroglobulin from human plasma (180 kD), β-galactosidase from Escherichia coli (116 kD), fructose-6-phosphatase from rabbit muscle (84 kD), pyruvat kinase from chicken muscle (58 kD), and fumarase from porcine heart (48.5 kD).

Isoelectric Focusing

Proteins were separated by IEF as described by van den Bosch et al. (1988) with some modifications. TfR and DPPIV bound to streptavidin agarose were eluted with 25 µl of 50 mM DL-dithiothreitol in dilution buffer, mixed with 8 µl of 8% (mass/vol) SDS, 34.8% (by vol) glycerol, 20% (by vol) mercaptoethanol, 250 mM Tris/HCl, pH 6.8, boiled for 3 min at 95°C, cooled, and added to 50 µl of urea sample buffer (9.5 M urea, 2% (by vol) NP-40, 2% (by vol) ampholines (40%, pH 3.5–9.5), 97 mM DL-dithiothreitol). Vertical 4% polyacrylamide gels containing 9.0 M urea, 2% (by vol) NP-40, 6% of a mixture of 40% ampholines (pH 3.5–9.5),

0.05% (by vol) TEMED and 0.02% (mass/vol) ammonium persulfate, were run for 15 min at 200 V, for 30 min at 300 V, and for 1 h at 400 V, using 20 mM H_3PO_4 as anodic buffer in the lower chamber and 50 mM NaOH as cathodic buffer in the upper chamber. Thereafter, samples were applied to the gel, overlaid with 4.75 M urea, 2% (by vol) NP-40, 1% (by vol) ampholines (40%; pH 3.5–9.5), 49 mM DL-dithiothreitol, and the gels were run for additional 18 h at 400 V. Gels were then processed for fluorography according to Bonner and Laskey (1974).

Carbohydrate Constituent Analysis

Glycoproteins (300 µg) were hydrolyzed for 1 h at 100°C in 1 N HCl. The resulting samples were analyzed by HPLC according to Lohmander (1986) on a HPX-87H column (300 mm × 7.8 mm; Bio-Rad, München, Germany) using 0.01 N sulfuric acid as solvent at a flow rate of 0.6 ml/min and a temperature of 65°C. Fractions (300 µl) were counted in a Packard Tri-Carb 1500 liquid scintillation system. N-Acetylneuraminic acid, L-fucose, D-mannose, D-galactose, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine served as internal standards.

Treatment of Glycoproteins with Glycosidases

Immunoadsorbed TfR and DPPIV were eluted from protein A–Sepharose by boiling at 95°C for 5 min in 0.4% (mass/vol) SDS, 5% (by vol) mercaptoethanol, and 40 mM EDTA, and were diluted fourfold in the appropriate glycosidase digestion buffer. Biotinylated proteins bound to streptavidin agarose were eluted with 50 mM DL-dithiothreitol in dilution buffer, concentrated by ultrafiltration in a Centricon-10 microconcentrator (Amicon), and suspended in glycosidase digestion buffer. Samples were split into two portions. One of these was incubated with the respective glycosidase at 37°C for 16 h, whereas the other was mock-digested under identical conditions without enzyme. Before addition of the glycosidases, all samples (100 µl) were heated at 95°C for 5 min, and a mixture of protease inhibitors (leupeptin, antipain, chymostatin, and pepstatin [10 µg each]) was added after cooling. Digestion with 10 mU of Endo H was performed in 20 mM sodium phosphate buffer, pH 5.5, 0.1% (by vol) NP-40, that with 10 mU of PNGase F in 20 mM sodium phosphate buffer, pH 7.5, 0.1% (by vol) NP-40, and that with 10 mU neuraminidase from *Vibrio cholerae* in 20 mM sodium phosphate, pH 5.5, 4 mM $CaCl_2$.

Treatment of Cells with Neuraminidase

Cells (3 × 10^6 in 100 mm-dishes) were cooled on ice, washed three times with ice-cold PBS, pH 5.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and were then incubated with 200 mU of neuraminidase (from *Clostridium perfringens*) in 4 ml PBS, pH 5.5, 4 mM $CaCl_2$, 1 mM $MgCl_2$ for 2 h at 4°C on a gently rocking platform. After washing five times with PBS/Ca^{2+}/Mg^{2+} cells were either recultured or harvested for further analysis. Desialylation of cell surface TfR required the prior removal of receptor-bound transferrin (Tf). Dissociation of the surface TfR–Tf complex was achieved adapting a procedure described by Dautry-Varsat et al. (1983). After washing cells were incubated at 4°C with deferoxamine mesylate (0.4 mM in PBS, pH 5.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$), for 10 min and washed once with 0.05 mM deferoxamine mesylate in the same buffer, to dissociate and to chelate iron from Tf at an acidic pH. Apo-Tf was then dissociated from the receptor by incubation of cells for 5 min in 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05 mM deferoxamine mesylate, followed by two washing cycles in the same buffer.

Determination of the Rate of Endocytosis

Rates of endocytosis were measured with a method similar to that described by Bretscher (1989) and Green and Kelly (1992). Cells were pulse-labeled with L-[^35S]methionine for 4 h, chased for 2 h, surface-labeled with NHS-SS-biotin at 4°C, and were then recultured at 37°C. At different times of reculture, the biotin label was removed by treating cells with the membrane impermeant thiol glutathione (GSH) at 4°C. Cell surface proteins that are internalized to intracellular compartments thereby acquire resistance to GSH, and, hence, retain the biotin label. Two dishes were frozen immediately after biotinylation without GSH treatment, to determine the total amount of biotinylated TfR and DPPIV. Two other dishes were treated with GSH immediately after biotinylation to determine the level of biotin label remaining bound to the proteins after GSH reduction. For treatment with GSH, cells were cooled to 4°C, washed three times with PBS/Ca^{2+}/Mg^{2+}, and incubated twice with freshly prepared 190 mM GSH in 75 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 290 mM NaOH, and

0.1% BSA (mass/vol), for 20 min each on a rocking platform. Subsequently, dishes were washed twice with PBS/Ca^{2+}/Mg^{2+}, incubated with 50 mM iodoacetamide in 75 mM Tris/HCl, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$ for 30 min at 4°C, and washed four times with PBS/Ca^{2+}/Mg^{2+}. Cells were harvested, detergent-extracted, and TfR and DPPIV were immunoadsorbed. The biotinylated forms of the two proteins were isolated on streptavidin agarose, subjected to SDS-PAGE and fluorographed. The films were scanned and the rates of internalization were calculated from the time-dependent increase of biotinylated TfR and DPPIV. The total amount of biotin-labeled TfR and DPPIV was determined from the dishes harvested immediately after biotinylation without being treated with GSH, and was taken as 100%. The amount of surface exposed protein retaining biotin label after treatment with GSH was determined from the dishes treated with GSH immediately after biotinylation, and was subtracted.

Half-lives of Biotinylated and Nonbiotinylated Surface Proteins

Cells were pulse-labeled with L-[^35S]methionine for 4 h and chased for 2 h. For determination of half-lives of the biotinylated form of TfR and DPPIV, cells were surface-labeled with NHS-SS-biotin immediately after the chase and were recultured for increasing periods of time (protocol a). For determination of half-lives of nonbiotinylated surface TfR and DPPIV, cells were surface-labeled with NHS-SS-biotin after reculture immediately before harvesting (protocol b). After detergent extraction of the cells, the biotinylated forms of TfR and DPPIV were isolated by immunoadsorption and binding to streptavidin agarose, separated by SDS-PAGE and fluorographed. Radioactivity of the polypeptide bands in the gel was quantitated by densitometry of fluorographs. Half-lives and rate constants of degradation were calculated from the decay of the radioactivity of the bands as described (Tauber et al., 1989).

Analysis of Recycling to the trans-Golgi Network

Cells were pulse labeled with L-[^35S]methionine for 4 h and chased for 2 h. Thereafter, cells were biotinylated at the surface at 4°C, treated with deferoxamine mesylate to dissociate Tf from cell surface TfR, and treated with neuraminidase (200 mU, *Clostridium perfringens*) at 4°C for 2 h on a gently rocking platform (details see above). After washing five times with ice-cold PBS/Ca^{2+}/Mg^{2+} to remove neuraminidase, cells were recultured in complete DME supplemented with 1 mM L-methionine. At different times of reculture aliquots of cells were detergent extracted, and TfR and DPPIV were immunoadsorbed. Biotinylated forms of the two proteins were isolated on streptavidin agarose, separated by IEF and fluorographed.

To analyze recycling of asialo-Tf to the TGN, cells (3 × 10^6 in 100-mm dishes) were preincubated for 30 min with 4 ml of DME containing 100 µM asialofetuin, 650 µM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid. Incubation was continued after addition of Fe^{3+}-loaded ^125I-asialo-Tf (1.5 µg/4 ml, 4 kBq/µg) to the culture medium. At different times, Tf in the culture medium was separated by IEF for analysis of resialylation. Fe^{3+}-Loaded asialo-Tf was prepared in that Tf was loaded with Fe^{3+} according to Karin and Mintz (1981), and was then labeled with ^125I sodium iodide by the method of Fraker and Speck (1978) to a specific radioactivity of 4 kBq/µg. ^125I-Tf (1.5 µg) was desialylated by digestion for 12 h at 20°C with 40 mU neuraminidase (*Clostridium perfringens*) immobilized to agarose (Sigma) and suspended in 200 µl PBS, pH 5.5, 4 mM $CaCl_2$, containing antipain, pepstatin, chymostatin, and leupeptin at a final concentration of 20 µg/ml each.

Analysis of Recycling to the trans/medial-Golgi Apparatus

Cells were cooled on ice, biotinylated at the surface, washed extensively to remove unreacted NHS-SS-biotin, and warmed to 37°C by addition of prewarmed complete DME. After addition of dMM to a final concentration of 1 mM and preincubation for 30 min, L-[6-^3H]fucose (15 MBq/3 × 10^6 cells) was added, and the incubation was continued for 6 h or 12 h, all in the presence of dMM. Cells were then washed four times with ice-cold PBS/Ca^{2+}/Mg^{2+}, and detergent extracted. For analysis of incorporation of L-[6-^3H]fucose into total cell surface proteins, biotinylated proteins were isolated from the homogenate on streptavidin agarose. For analysis of incorporation of L-[6-^3H]fucose into biotinylated TfR and DPPIV, the two proteins were immunoadsorbed, and the biotinylated forms were isolated

by affinity chromatography on streptavidin agarose, separated by SDS-PAGE, and fluorographed.

Analysis of Recycling to the cis-Golgi Apparatus

Cells were preincubated with 1 mM dMM in MEM without L-methionine/cysteine for 30 min at 37°C, followed by addition of L-[35S]methionine (5.5 MBq/3 × 10⁶ cells) and further incubation for 4 h. Cells were then washed three times with complete DME supplemented with 1 mM L-methionine, and incubated for further 2 h all in the presence of 1 mM dMM. After cooling on ice, cells were surface-biotinylated at 4°C, washed four times to remove unreacted NHS-SS-biotin, rewarmed to 37°C by addition of complete DME without dMM, but supplemented with 1 mM L-methionine, and recultured. An aliquot of the cells was recultured in the presence of 1 mM dMM. After 12 h cells were detergent extracted, and the biotinylated forms of TfR and DPPIV were isolated by immunoadsorption and affinity chromatography on streptavidin agarose, separated by SDS-PAGE and fluorographed.

Susceptibility of Oligomannosidic Cell Surface DPPIV to Golgi Mannosidases

After preincubation with 1 mM dMM for 30 min, HepG2 cells were labeled with D-[2-³H]mannose (18 MBq/3 × 10⁶ cells) for 16 h in the presence of 1 mM dMM and were thereafter surface-labeled with NHS-SS-biotin. From these cells a membrane fraction containing the plasma membrane was isolated and divided into two portions. One portion (3 mg protein) was incubated with Golgi apparatus isolated from rat liver. For this, isolated Golgi apparatus (3.5 mg protein) and the isolated membranes from [³H]mannose-labeled HepG2 cells were mixed in a final volume of 4 ml Golgi buffer (100 mM NaAc, pH 6.0, 0.1% NP-40 [by vol]), resuspended by 15 strokes in a Dounce homogenizer, sonified for 30 s using a Branson B-R sonifier (Branson Ultrasonics Corp., Danbury, CT), and incubated at 37°C for 16 h. The other portion (2 mg) was treated likewise, but without isolated Golgi membranes. After incubation membranes were solubilized by addition of NP-40 to a final concentration of 1% (by vol). Insoluble material was removed by ultracentrifugation (30 min, 100,000 g). From the supernatants the biotinylated form of DPPIV representing cell surface DPPIV was isolated by immunoaffinity chromatography in conjunction with affinity chromatography on streptavidin agarose.

Analysis of Oligosaccharides by HPAE Chromatography

Oligosaccharides were released from isolated DPPIV by PNGase F and separated from residual proteins by chloroform/methanol precipitation. The samples (300 μl) were mixed thoroughly with 600 μl methanol, 100 μl chloroform, and 100 μl water, and centrifuged at 10,000 g for 5 min. The upper phase was collected, dried under vacuum, redissolved in 100 μl water and applied to a C18-cartridge (Waters Chromatography Div., Milford, MA) to remove traces of detergents. After washing with water the eluants (2 ml) were dried and desalted by gel filtration on Bio-Gel P-2 columns as previously described (Orberger et al., 1992). Fractions containing radioactivity were pooled, dried, redissolved in 100 μl water, and analyzed by HPAE chromatography. Separations were carried out using a Dionex (Sunnyvale, CA) BioLC system and a CarboPac PA-100 column (4 × 250 mm) in series with a CarboPac PA-100 guard column according to Pfeiffer et al. (1990). A 50% solution of NaOH (Baker, Gross-Gerau, Germany) and sodium acetate (Fluka, Buchs, Switzerland) were used to prepare the eluants (Eluant A, 200 mM NaOH in water; Eluant B, 200 mM NaOH, 250 mM sodium acetate in water). Columns were calibrated with authentic oligomannosidic oligosaccharides Man₅₋₉GlcNAc₂ isolated from thyreoglobulin and from ribonuclease (Yamamoto et al., 1981). Oligosaccharides were eluted at a flow rate of 1 ml/min using a linear gradient from 0 to 15% B within 45 min and collected in 1-ml fractions. After addition of scintillation cocktail (Packard Instruments, Meriden, CT) the fractions were monitored for radioactivity with a model 4450 liquid scintillation counter (Packard Instruments, Meriden, CT).

Results

Glycosylation of TfR and DPPIV in HepG2 Cells

In order to characterize N-glycosylation of TfR and DPPIV

in HepG2 cells, both glycoproteins were immunoadsorbed from cells metabolically labeled with L-[35S]methionine and were digested with either Endo H, PNGase F, or neuraminidase (Fig. 1). Digestion of TfR with Endo H, cleaving N-glycans of the high mannose and the hybrid type (Trimble and Maley, 1984), reduced the apparent molecular mass of TfR from 95 to 90 kD, while digestion with PNGase F, cleaving all types of N-glycans (Tarentino et al., 1985), resulted in the formation of a 86-kD polypeptide. Neuraminidase reduced the apparent molecular mass of TfR from 95 to 93 kD. This indicates that TfR in HepG2 cells is sialylated and has N-glycans of the high mannose or the hybrid type and of the complex-type, in accordance with results obtained in several different human lymphoblastoid cell lines (Omary and Trowbridge, 1981; Schneider et al., 1982), and with the glycan structure analysis of TfR from HepG2 cells (Orberger et al., 1992).

DPPIV was resistant to Endo H, but was sensitive to both PNGase F and neuraminidase, reducing the apparent molecular mass from 125 to 95 and 115 kD, respectively. Hence, N-glycans of DPPIV in HepG2 cells are mainly, if not entirely of the sialylated complex type. Likewise, TfR and DPPIV could be metabolically labeled with L-[6-³H]fucose, D-[2-³H]mannose and D-[2-³H]galactose (data not shown). As shown in pulse–chase experiments, oligosaccharide processing of TfR and DPPIV occurred within 60 to 120 min (data not shown).

Surface Labeling of HepG2 Cells with NHS-SS-Biotin

Surface proteins of HepG2 cells were labeled with NHS-SS-biotin and were separated from nonbiotinylated proteins by affinity chromatography on streptavidin agarose. As has been demonstrated recently, labeling with NHS-SS-biotin is selective for cell surface proteins of hepatoma cells and hepatocytes under the conditions applied (Busch et al., 1989; Loch et al., 1992). The biotinylated form both of total cell surface proteins (Fig. 2 A) and of surface TfR and DPPIV (Fig. 2 B) could be separated from the nonbiotinylated proteins. Proteins from nonbiotinylated cells were not bound to streptavidin agarose (Fig. 2 A).

To determine the portion of TfR and DPPIV, that could be biotinylated at the cell surface and isolated by affinity chromatography on streptavidin agarose, aliquots of total TfR and DPPIV obtained by immunoadsorption from the detergent extracts, and of the biotinylated species eluted from streptavidin agarose were separated by SDS-PAGE, fluorographed and quantified densitometrically. About
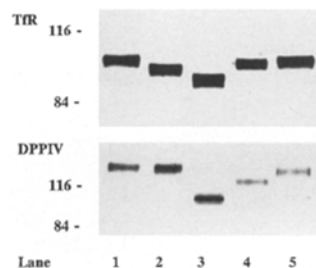

Figure 1. Digestion of TfR and DPPIV with Endo H, PNGase F and neuraminidase. DPPIV and TfR were immunoadsorbed from detergent extracts of HepG2 cells metabolically labeled for 2 h with L-[35S]methionine (5.5 MBq/3 × 10⁶ cells) and chased for 2 h. The isolated proteins were split into five portions and were either mock-digested (lanes 1 and 5) or were treated with Endo H (lane 2), PNGase F (lane 3), or neuraminidase (lane 4) as detailed in Material and Methods.

A

B
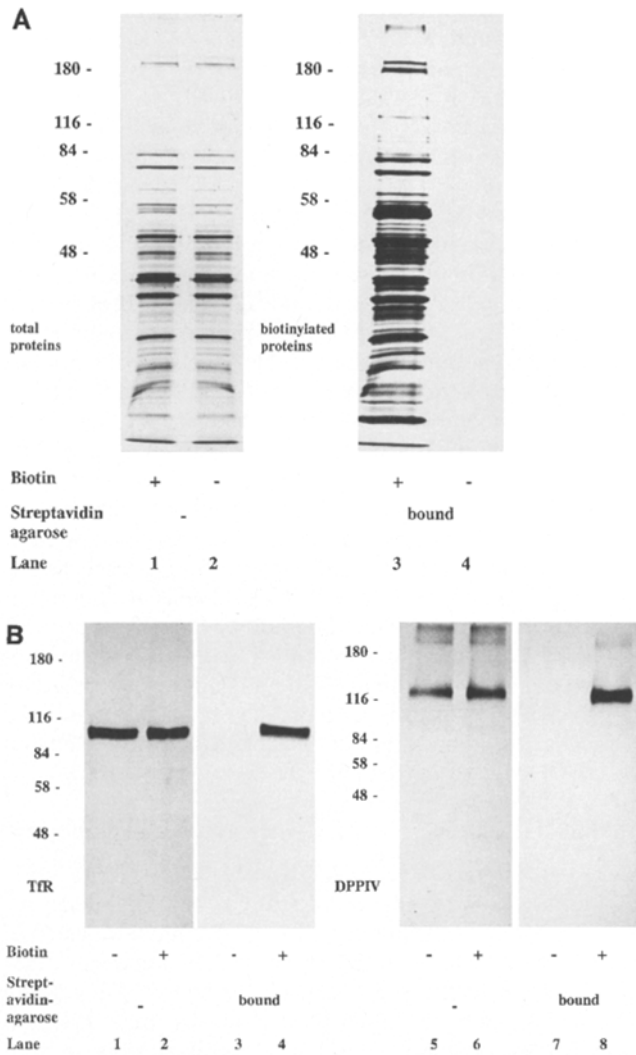
Figure 2. Separation of biotinylated and nonbiotinylated proteins of HepG2 cells on streptavidin agarose. (A) Total cell proteins. HepG2 cells were surface labeled with NHS-SS-biotin at 4°C (lanes 1 and 3), or were mock-treated (lanes 2 and 4) as described in Material and Methods. Detergent extracts from both biotinylated and nonbiotinylated cells were subjected to affinity chromatography on streptavidin agarose as detailed in Material and Methods. Protein samples obtained before chromatography (lanes 1 and 2) and proteins eluted with DTT (lanes 3 and 4) were separated by SDS-PAGE and were silver stained. (B) TfR and DPPIV. HepG2 cells were labeled with L-[^{35}S]methionine (5.5 MBq/3 × 10⁶ cells, 4 h pulse, 2 h chase) and were either biotinylated at the cell surface or mock-treated as in A. TfR and DPPIV were immunoadsorbed from detergent extracts and were subjected to streptavidin agarose chromatography. Samples obtained before chromatography and after elution with DTT were separated on 7.5% SDS–polyacrylamide gels and were fluorographed. TfR and DPPIV from biotinylated (lanes 2, 4, 6, and 8) and nonbiotinylated cells (lanes 1, 3, 5, and 7), before (lanes 1, 2, 5, and 6), and after (lanes 3, 4, 7, and 8) separation on streptavidin agarose.

20% of total cellular TfR and 60% of total cellular DPPIV could be biotinylated and retrieved from the affinity matrix (not shown). This was highly reproducible in all the experiments.

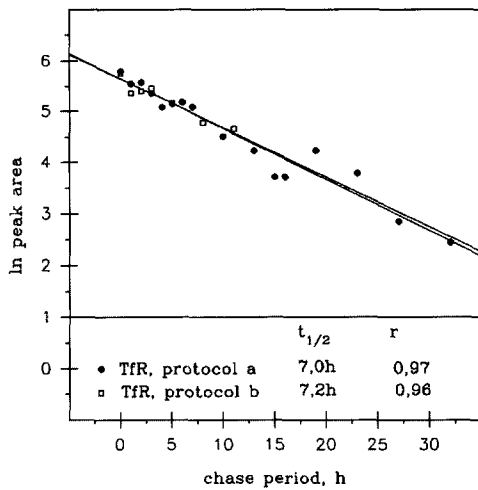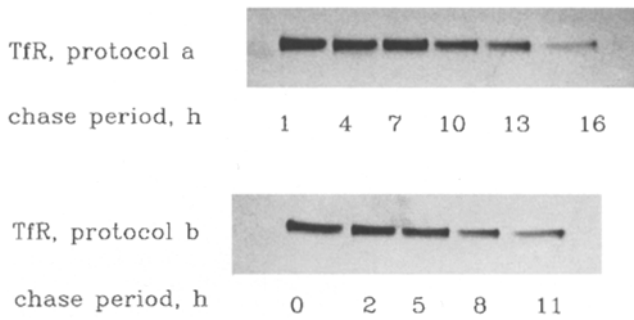To exclude that biotinylation influences the metabolic stability of the proteins, half-lives of biotinylated and nonbiotinylated surface TfR and DPPIV were compared. For determination of the half-life of the nonbiotinylated form of surface TfR and DPPIV, HepG2 cells were pulse–chase labeled with L-[^{35}S]methionine for 4 h and were further cultured for up to 32 h. At different times surface proteins were isolated in that cells were cooled to 4°C, surface-labeled with biotin, and detergent extracted. Biotinylated, i.e., surface exposed proteins were then isolated by streptavidin agarose chromatography. In comparison, the stability of biotinylated surface TfR and DPPIV was measured in cells that were surface-labeled with biotin immediately after the pulse/chase and were thereafter recultured for different periods of time. For determination of half-lives proteins were separated by SDS-PAGE and fluorographed. Half-lives were calculated from the decay of the relative intensities of the radiolabeled polypeptide bands (Fig. 3). Both, the biotinylated and the nonbiotinylated form of surface TfR were degraded with the same half-life of 7.0 h and 7.2 h, respectively, indicating that biotinylation did not affect the stability of the glycoprotein. Likewise, biotinylation did not affect the stability of DPPIV (not shown) that was degraded with a half-life of about 30 h in the biotinylated and nonbiotinylated form.

Rates of Internalization of TfR and DPPIV

Rates of internalization of TfR and DPPIV were determined using the following experimental design. After metabolic labeling with L-[^{35}S]methionine cells were labeled with NHS-SS-biotin at 4°C and were recultured at 37°C to allow surface proteins to be internalized. At different times of reculture, cells were cooled to 4°C, and biotin residues remaining on the cell surface were removed by reduction with GSH. GSH treatment of cells at 4°C quantitatively removed biotin residues exposed on the cell surface as was assured beforehand and in parallel cultures in each experiment. In these control experiments, cells were treated with GSH immediately after biotinylation. No or only traces of biotin label remained on the cells after GSH treatment. Proteins retaining biotin label after GSH treatment, hence, represent molecules that have been internalized into intracellular compartments. As shown in Fig. 4, 50–60% of surface TfR were internalized within 15 min of incubation. This percentage slowly increased during further incubation of cells most likely because TfR requiring ∼15 min for one cycle (Klausner et al., 1983) reappears on the cell surface where it becomes sensitive to GSH treatment again. After 60 min of reculture ∼80% of surface TfR had become GSH-resistant, in accordance with previous studies using other experimental approaches (Klausner et al., 1983; Rothenberger et al., 1987; Collawn et al., 1993). DPPIV became GSH resistant to a much lower extent. After 4 h of incubation at 37°C, only 15% of cell surface DPPIV was internalized (Fig. 4).

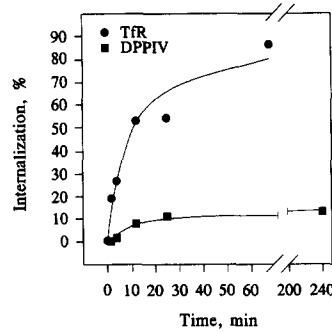## Surface TfR and Surface DPPIV Do Not Significantly Return to Mannosidase I in the cis-Golgi

Covalent labeling of surface proteins with NHS-SS-biotin in conjunction with the experimental strategy, initially described by Snider and Rogers (1986), Duncan and Kornfeld (1988), and Neefjes et al. (1988) was used to study

TfR, protocol a

chase period, h   1   4   7   10   13   16

TfR, protocol b

chase period, h   0   2   5   8   11

Figure 4. Kinetics of internalization of TfR and DPPIV in HepG2 cells. Cells were labeled with L-[^{35}S]methionine for 4 h (5.5 MBq/3 × 10^6 cells) and chased for 1 h. After surface labeling with NHS-SS-biotin at 4°C, cells were recultured at 37°C for the times indicated. Cells were then cooled to 4°C, treated with GSH as described in Material and Methods and detergent extracted. DPPIV and TfR were immunoadsorbed from the detergent extracts. The biotinylated forms of the two proteins were isolated by affinity chromatography on streptavidin agarose, separated by SDS-PAGE and fluorographed. The fluorograms were densitometered, and the percentage of internalization of TfR and DPPIV was calculated as detailed in Material and Methods.

Figure 3. Half-lives of biotinylated and nonbiotinylated surface TfR in HepG2 cells. Surface TfR in nonbiotinylated cells (protocol a): HepG2 cells were labeled for 4 h with L-[^{35}S]methionine (5.5 MBq/3 × 10^6 cells) and chased. At the times indicated, cells were cooled to 4°C, surface-labeled with NHS-SS-biotin, and detergent extracted. Surface TfR in biotinylated cells (protocol b): Cells were pulse labeled with L-[^{35}S]methionine as in *a* and chased for 2 h. Thereafter, cells were cooled to 4°C, surface labeled with NHS-SS-biotin, rewarmed to 37°C and were further cultured for the times indicated. At the different time points, cells were detergent extracted. For determination of half-lives TfR was immunoadsorbed from detergent extracts and subjected to affinity chromatography on streptavidin agarose. Biotinylated TfR was eluted with DTT, separated by SDS-PAGE and fluorographed (*top*). Fluorographs were scanned and peak areas were plotted semilogarithmically against time (*bottom*). Half-lives ($t_{1/2}$) were calculated from the slope of the regression line, and correlation coefficients (*r*) were calculated. Half-lives are the means of three independent experiments.

transport of TfR and DPPIV from the cell surface to *cis*-Golgi cisternae. Using this experimental design it was possible to distinguish recycling surface glycoproteins from newly synthesized proteins en route to the cell surface.

Mannosidase I of the *cis*-Golgi is reversibly inhibited by dMM (Bischoff and Kornfeld, 1984; Elbein et al., 1984; Fuhrmann et al., 1984). As a consequence, the *N*-glycans of newly synthesized glycoproteins normally processed to the complex type, retain high-mannose structures. These nonprocessed forms of the glycoproteins were labeled metabolically in pulse–chase experiments. Upon transport to

the cell surface, these glycoproteins were additionally surface labeled with NHS-SS-biotin. Thereafter, inhibition of mannosidase I was reversed by washout of the inhibitor. Biotinylated cell surface glycoproteins with high mannose oligosaccharides that subsequently return to the *cis*-Golgi complex should be trimmed by mannosidase I and further processed to glycoproteins with complex-type glycans. Conversion of high-mannose oligosaccharides to complex-type structures in glycoproteins labeled with both [^{35}S]methionine and biotin would, hence, report recycling of these glycoproteins from the cell surface to the *cis*-Golgi compartment.

dMM at a concentration of 1 mM inhibited *N*-glycan processing of DPPIV and TfR. DPPIV and TfR labeled in the presence of dMM (Fig. 5, lane *3*) appeared on the cell surface with intensities similar to the intensities of DPPIV and TfR labeled in the absence of the inhibitor (lane *1*) indicating that dMM does not inhibit transport of newly synthesized DPPIV and TfR to the cell surface, in accordance with other reports (Neefjes et al., 1988; Loch et al., 1992). Both glycoproteins had a reduced apparent molecular mass of 110 and 90 kD, respectively, when labeled in the presence of dMM (lanes *3* and *8*) compared to a molecular mass of 125 and 95 kD, respectively, observed in the absence of the inhibitor (lanes *1* and *7*). Likewise, both glycoproteins were Endo H sensitive when labeled in the presence of dMM (lane *4*), but were either fully resistant to Endo H as for DPPIV or partly resistant to Endo H as for TfR in the absence of dMM (lane *2*).

To test reversibility of the inhibition of mannosidase I by dMM, HepG2 cells were incubated with dMM for 6 h, followed by washout of the inhibitor and metabolic labeling. TfR and DPPIV synthesized under these conditions (Fig. 5, lanes *5*, *6*, and *9*) exhibited the same apparent molecular mass and the same Endo H resistance as those from untreated control cells (lanes *1*, *2*, and *7*).

To find out whether TfR and DPPIV return from the cell surface to *cis*-Golgi cisternae, both glycoproteins were pulse-chase labeled with L-[^{35}S]methionine in the presence of dMM and were biotinylated on the cell surface. Cells were then recultured after washout of the inhibitor and the biotinylated forms of TfR and DPPIV were isolated
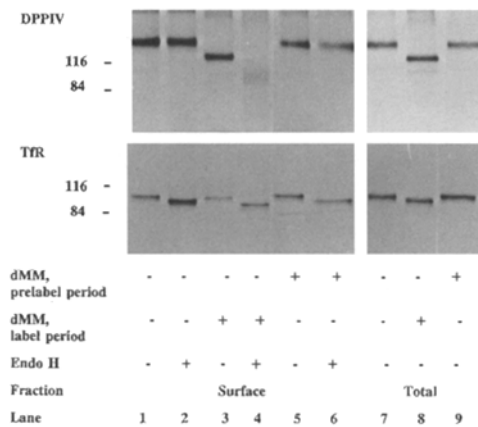
Figure 5. Reversibility of mannosidase I inhibition by dMM. For demonstration of the effect of dMM, cells were incubated with 1 mM dMM for 30 min and were then labeled with L-[^{35}S]methionine (5.5 MBq/3 × 10^6 cells) for 4 h, followed by a 2 h chase. Presence of 1 mM dMM was maintained during the pulse and chase period (lanes *3, 4*, and *8*). Controls were pulse-chase labeled for the same times without dMM (lanes *1, 2*, and *7*). To prove reversibility of inhibition by dMM, cells were incubated for 6 h with 1 mM dMM, followed by washout of the inhibitor (see Material and Methods) and metabolic labeling with L-[^{35}S]methionine (4 h pulse, 2 h chase, lanes *5, 6*, and *9*). Cells were then surface-labeled with NHS-SS-biotin. Total cellular DPPIV and TfR were immunoadsorbed from detergent extracts (total fraction, lanes *7–9*). The surface exposed, i.e., biotinylated forms of TfR and DPPIV (lanes *1–6*) were isolated from the total fraction by affinity chromatography on streptavidin agarose. Proteins eluted from streptavidin agarose with DTT were split into two portions, one of which was digested with Endo H (lanes *2, 4*, and *6*), while the other was mock-digested (lanes *1, 3*, and *5*).

and subjected to gel electrophoresis. For comparison, in parallel cultures presence of dMM was maintained during reculture, whereas a third portion of the cells was labeled, biotinylated and recultured in the absence of the inhibitor. When compared to the processed forms of TfR and DPPIV synthesized in the absence of the inhibitor (Fig. 6, lane 2), both glycoproteins, even after 12 h of reculture in the absence of dMM retained the reduced molecular mass (lanes *1* and *4*) that was observed when the presence of dMM was maintained during reculture (lane *3*). Reprocessed forms of cell surface DPPIV and of TfR were not detectable even after extreme overexposure of the gels. This indicates that DPPIV and TfR do not significantly recycle from the cell surface to the *cis*-Golgi compartment.

Susceptibility of Oligomannosidic Cell Surface DPPIV to Golgi Mannosidases

The result that the biotinylated oligomannosidic glycoform of the two cell surface glycoproteins is not converted to the complex type glycoform, indicating exclusion of transport to the *cis*-Golgi, could also reflect resistance of the biotinylated, oligomannosidic surface type of the glycoproteins to Golgi mannosidases. It was, therefore, necessary to prove that the biotinylated oligomannosidic glycoform of surface DPPIV can be trimmed by Golgi mannosidases in principle. To this end, this species of DP-PIV was incubated in vitro with isolated Golgi apparatus and was, thereafter, analyzed with respect to trimming of
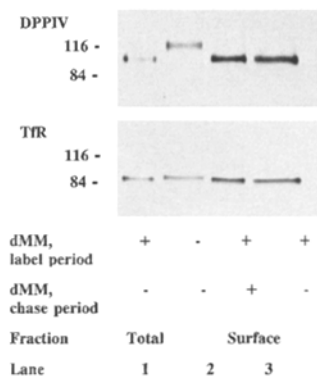
Figure 6. Return of TfR and DPPIV to *cis*-Golgi cisternae. HepG2 cells were preincubated for 30 min with 1 mM dMM and were pulse-chase labeled (4 h pulse, 2 h chase, 5.5 MBq/3 × 10^6 cells) with L-[^{35}S]methionine, all in the presence of dMM. Thereafter, cells were biotinylated at the cell surface at 4°C, and dMM was washed out. After rewarming to 37°C cells were recultured for further 12 h either in the presence (lane *3*) or absence (lanes *1* and *4*) of dMM in parallel cultures. Control cells were labeled, biotinylated and recultured in the absence of dMM (lane *2*). TfR and DPPIV were immunoadsorbed from detergent extracts of the cells. Biotinylated forms (lanes *2–4*) were isolated by affinity chromatography on streptavidin agarose, separated by SDS-PAGE and fluorographed. Lane *1* represents an aliquot of immunoadsorbed TfR and DPPIV before chromatography on streptavidin agarose.

its oligomannosidic oligosaccharides. Cells were metabolically labeled with D-[2-^{3}H]mannose in the presence of dMM, followed by derivatization of cell surface proteins with NHS-SS-biotin. A membrane fraction containing surface membranes was prepared from the labeled cells and was then incubated with isolated Golgi apparatus. Biotiny-
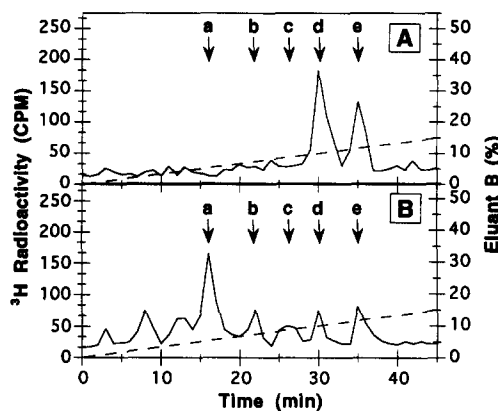
Figure 7. Susceptibility of oligomannosidic glycans of biotinylated cell surface DPPIV to Golgi mannosidases. HepG2 cells were preincubated for 30 min with 1 mM dMM and were metabolically labeled with D-[2-^{3}H]mannose (16 h, 18 MBq/3 × 10^6 cells) in the presence of dMM. After biotinylation of surface proteins membranes were isolated and incubated with isolated Golgi apparatus as detailed in Materials and Methods. A control was treated likewise in the absence of Golgi apparatus. Biotinylated DPPIV was isolated from both preparations and glycans were released by PNGase F. After removal of residual proteins by chloroform/methanol precipitation and desalting on a BioGel P-2 column glycans were fractionated by HPAE chromatography using a Carbo-Pac PA-100 column (4 × 250 mm) and a linear gradient of 250 mM sodium acetate in 200 mM NaOH. Fractions (1 ml) were collected at 1 ml/min and monitored for radioactivity. Glycans obtained from DPPIV incubated in the absence (*A*) or presence (*B*) of Golgi apparatus are shown. Arrows in A and B indicate the elution times of oligomannosidic oligosaccharide standards with Man$_5$-GlcNAc$_2$ (*a*), Man$_6$GlcNAc$_2$ (*b*), Man$_7$GlcNAc$_2$ (*c*), Man$_8$GlcNAc$_2$ (*d*), and Man$_9$GlcNAc$_2$ (*e*).

lated DPPIV was isolated from the membranes by immunoaffinity chromatography in conjunction with affinity chromatography on streptavidin agarose. Oligosaccharides were released from DPPIV by PNGase F and analyzed by HPAE chromatography. Cell surface DPPIV, which was not incubated with Golgi membranes was glycosylated with $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ in a ratio of 2:3 (Fig. 7 A), in line with the inhibitory effect of dMM on ER- and Golgi mannosidases (for review see Daniel et al., 1994). After incubation with Golgi membranes >75% of $Man_{8-9}GlcNAc_2$ was trimmed to smaller sized oligosaccharides (Fig. 7 B). Comparison with authentic oligomannosidic oligosaccharides used as internal standards showed that the smaller sized glycans included $Man_{5-7}GlcNAc_2$ with $Man_5GlcNAc_2$ being the main product. With respect to the reported properties of α-mannosidases (for review see Daniel et al., 1994), generation of the observed pattern of trimming products should involve the action of mannosidase I. Hence, biotinylated cell surface DPPIV can be trimmed by Golgi mannosidases. In summary, the inability to demonstrate return of cell surface DPPIV to the *cis*-Golgi does not reflect in principle resistance of the biotinylated high mannose type glycoform of DPPIV to Golgi mannosidases.
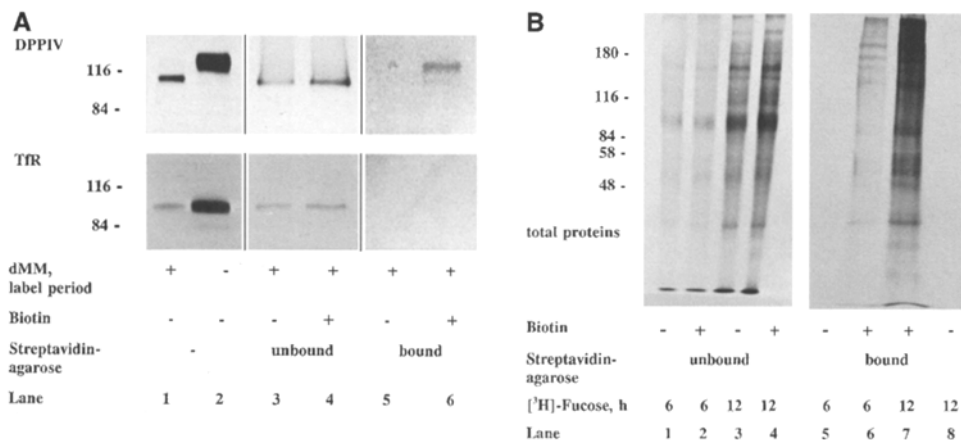
Surface DPPIV, but Not Surface TfR Returns to Sites of Fucosyltransferases

Surface glycoproteins continuously loose fucose residues during recycling and, hence, have acceptor sites for new fucose residues (Tauber et al., 1983; Kreisel et al., 1988). To analyze return of surface glycoproteins to sites of fucosyltransferases, surface proteins of HepG2 cells cultured under standard conditions were biotinylated at 4°C followed by reculture of the cells at 37°C in the presence of $L-[6-^3H]$fucose (Fig. 8). Incorporation of $L-[^3H]$fucose into the biotinylated form of a glycoprotein should report return of this glycoprotein from the cell surface to sites of fucosyltransferases. Since it had to be expected that during reculture [³H]fucose was primarily incorporated into newly synthesized glycoproteins en route to the cell surface, it was essential to unambiguously differentiate incorporation of [³H]fucose into preformed surface glycoproteins (recycling to the Golgi apparatus) from that into newly synthesized glycoproteins (trafficking through the Golgi apparatus en route to the cell surface). In addition to labeling surface proteins with biotin as the first criterion for differentiation we, therefore, employed the differences in the molecular mass of preformed and of newly synthesized glycoproteins as a second criterion for differentiation. To accomplish this, reculture of cells with $L-[^3H]$fucose was performed in the presence of dMM. In the presence of dMM, newly synthesized proteins incorporating $L-[^3H]$fucose were not processed and, hence, retained a reduced apparent molecular mass. For DPPIV, the nonprocessed form showed an apparent molecular mass of 110 kD, and could be easily distinguished from the preformed DPPIV generated in the absence of dMM and, therefore, having an apparent molecular mass of 125 kD (Fig. 9 A, lanes 1 and 2). In the recycling experiments preformed cell surface DPPIV and TfR generated in the absence of dMM, and, hence, having the higher molecular mass were biotinylated and were then monitored for incorporation of [³H]fucose. Incorporation of [³H]fucose into this species of the two glycoproteins should, therefore, report return of surface DPPIV or TfR to the sites of fucosyltransferases,
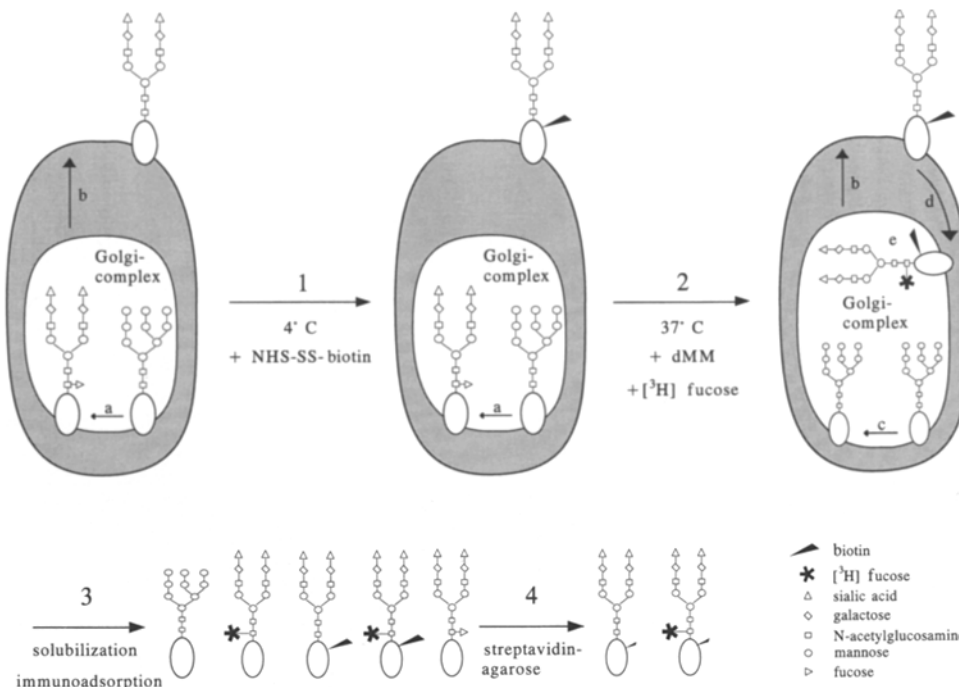
Figure 8. Experimental design to study return of surface TfR and DPPIV to sites of fucosyltransferases. (*1*) Cell surface glycoproteins of HepG2 cells were biotinylated at 4°C. (*2*) After warming to 37°C cells were recultured with [³H]fucose in the presence of 1 mM dMM. (*3*) DPPIV and TfR were isolated from detergent extracts of cells by immunoadsorption. (*4*) The biotinylated surface form of DPPIV and TfR were isolated by affinity chromatography on streptavidin agarose and analyzed by SDS-PAGE and fluorography. (*a*) Oligosaccharide processing of newly synthesized glycoproteins, (*b*) surface transport, (*c*) inhibition of oligosaccharide processing of newly synthesized glycoproteins by dMM, (*d*) internalization, (*e*) transfer of ³H-fucose to surface glycoproteins recycling to the Golgi complex.

A

DPPIV

116 -
84 -

TfR

116 -
84 -

dMM, label period	+	-	+	+	+	+
Biotin	-	-	-	+	-	+
Streptavidin-agarose		-	unbound		bound	
Lane	1	2	3	4	5	6

B

180 -
116 -
84 -
58 -
48 -

total proteins

Biotin	-	+	-	+	-	+	+	-
Streptavidin-agarose		unbound				bound		
[³H]-Fucose, h	6	6	12	12	6	6	12	12
Lane	1	2	3	4	5	6	7	8

Figure 9. Return of surface glycoproteins to sites of fucosyltransferases. HepG2 cells were surface labeled with NHS-SS-biotin at 4°C, rewarmed to 37°C and recultured in the presence of L-[³H]fucose (15 MBq/3 × 10⁶ cells) and 1 mM dMM for 6 or 12 h. Controls for unspecific binding of proteins to streptavidin agarose were treated likewise, but without prior labeling with NHS-SS-biotin. (*A*) TfR and DPPIV. TfR and DPPIV were immunoadsorbed from detergent extracts and subjected to chromatography on streptavidin agarose as detailed in Material and Methods. Lanes *3* and *4*: nonbiotinylated DPPIV/TfR not bound to streptavidin agarose; lanes *5* and *6*: biotinylated DPPIV/TfR eluted from streptavidin agarose with DTT. To monitor the effect of dMM on incorporation of L-[³H]fucose into newly synthesized glycoproteins, nonbiotinylated cells were cultured with L-[³H]fucose in the presence (lane *1*) or absence of 1 mM dMM (lane *2*) for 6 h. (*B*) Total surface glycoproteins. Aliquots of detergent extracts were subjected to chromatography on streptavidin agarose. Lanes *1–4*: Aliquots of cell homogenates before affinity chromatography on streptavidin agarose. Lanes *5–8*: Proteins from biotin-labeled or mock-treated cells eluted from streptavidin agarose with DTT. Proteins were separated on 7.5% SDS–polyacrylamide gels and fluorographed.

whereas incorporation of [³H]fucose into the nonbiotinylated form of DPPIV or TfR with the lower molecular mass would reflect labeling of newly synthesized glycoproteins en route to the surface. When biotinylated DPPIV and TfR were analyzed after reculture of cells for 12 h in the presence of L-[³H]fucose, fucose was incorporated into the biotinylated 125-kD form of DPPIV, evidencing that cell surface DPPIV is recycled to Golgi fucosyltransferases (Fig. 9 *A*, lane *6*). In contrast, no incorporation of [³H]fucose into biotinylated TfR was detectable even after 6 mo of exposure of the film (Fig. 9 *A*, lane *6*). On the other hand, newly synthesized TfR was labeled with [³H]fucose to a similar extent as was newly synthesized DPPIV (Fig. 9, lane *2*). This demonstrates that our inability to detect fucosylation of recycling TfR cannot be explained by a low number of fucose residues on the receptor. This conclusion is further supported by the recent analysis of the structure of the N-linked oligosaccharides of TfR from HepG2 cells showing that the receptor is clearly fucosylated (Orberger et al., 1992). It is, therefore, concluded that cell surface TfR does not, or does only at insignificant amounts recycle to Golgi fucosyltransferases in HepG2 cells. Additional controls showed that no unspecific binding to streptavidin agarose was noted for the proteins from nonbiotinylated control cells (Fig. 9 *A*, lane *5*). Moreover, as expected, the newly synthesized form of DPPIV generated during 12 h of reculture excusively had an apparent molecular mass of 110 kD (Fig. 9 *A*, lanes *3* and *4*).

Incorporation of L-[³H]fucose into surface glycoproteins was not restricted to DPPIV. Analysis of the whole set of biotinylated proteins after reculture of cells with L-[³H]fucose for 6 h or 12 h, demonstrated that L-[³H]fucose was incorporated into several polypeptide bands with an apparent molecular mass in between 100 and 200 kD in a time-dependent manner (Fig. 9 *B*, lanes *6* and *7*). No incorporation was noted into proteins from non-biotinylated control cells, excluding unspecific binding of glycoproteins to streptavidin agarose (Fig. 9 *B*, lanes *5* and *8*).

As was assured by carbohydrate constituent analysis of

the glycoproteins, L-[³H]fucose was not metabolized to other radiolabeled monosaccharides during the experiment (not shown).

Surface DPPIV, but Not Surface TfR Is Resialylated in HepG2 Cells

In experiments designed to study return of surface TfR and DPPIV to *trans*-elements of the Golgi apparatus and to the TGN, proteins of HepG2 cells labeled with L-[³⁵S]methionine were biotinylated at the cell surface and thereafter desialylated with neuraminidase at 4°C to generate biotinylated asialo-TfR and asialo-DPPIV. The cells were then rewarmed to 37°C and were recultured for different periods of time. Resialylation of biotinylated asialo-TfR and asialo-DPPIV should report return of both glycoproteins from the cell surface to sites of sialyltransferases.

Beforehand, conditions for desialylation of the two glycoproteins on the surface of HepG2 cells with neuraminidase were established. As shown by IEF surface TfR could not be desialylated at 4°C even with high activities of neuraminidase (200 mU/3 × 10⁶ cells) (Fig. 10, lanes *1* and *2*). On the other hand, desialylation of TfR was achieved when digestion with neuraminidase was performed with intact cells at 37°C (Fig. 10, lane *3*) or with detergent extracts of the cell homogenate at 4°C (Fig. 10, lanes *4* and *5*). This indicated that at 4°C sialic acids residues of membrane-bound TfR on the cellular surface are not accessible to neuraminidase. Since TfR on HepG2 cells is almost completely saturated with Tf (Neefjes et al., 1990), we tried to increase accessibility of the TfR glycans for neuraminidase by removing receptor-bound Tf according to Dautry-Varsat et al. (1983). As shown in Fig. 10 (lanes *6* and *7*), surface TfR could indeed be desialylated at 4°C after removal of Tf. Likewise, surface DPPIV was desialylated by digestion of cells with neuraminidase, as indicated by the shift of the polypeptide bands from the acidic to the more basic part of the IEF gel (Fig. 11 *A*, lanes *2* and *3*).

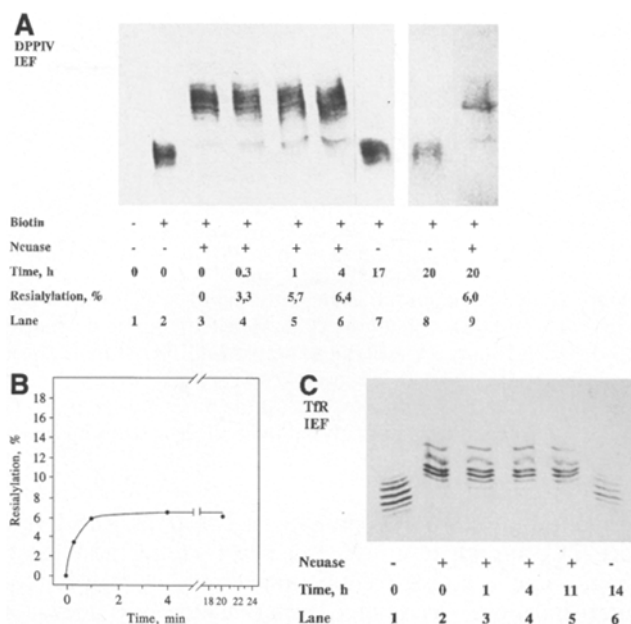When cells were recultured for increasing periods of
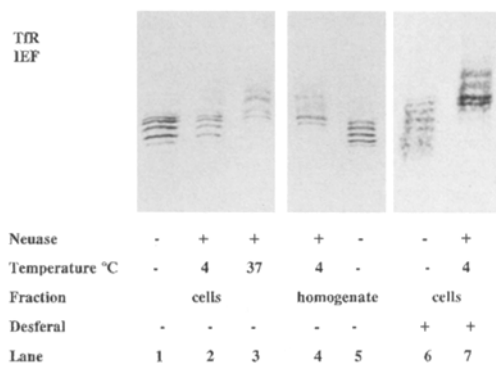
Figure 10. Desialylation of surface TfR with neuraminidase. HepG2 cells were labeled for 4 h with L-[^{35}S]methionine (5.5 MBq/3 × 10^6 cells) followed by a 2-h chase and biotinylation of surface proteins at 4°C. Intact cells were then incubated with neuraminidase (200 mU/3 × 10^6 cells) for 2 h at 4°C (lane 2), or 37°C (lane 3), or were incubated without neuraminidase for 2 h at 4°C (lane 1). Alternatively, 6 × 10^6 cells were homogenized. Homogenates were split into two portions that were either incubated with neuraminidase (50 mU) for 2 h at 4°C (lane 4), or were mock-digested (lane 5). Tf was removed from TfR by incubating the cells with deferoxamine mesylate as described in Material and Methods. Cells were then incubated with neuraminidase (200 mU/3 × 10^6 cells) for 2 h at 4°C (lane 7) or were mock-digested (lane 6). TfR was immunoadsorbed from detergent extracts and the biotinylated form of TfR was isolated by affinity chromatography on streptavidin agarose as detailed in Material and Methods. Samples were separated by IEF (basic side of the gel at the top) and were fluorographed.

time for up to 20 h after surface biotinylation, analysis of biotinylated DPPIV by IEF showed that the isoelectric point of a small portion of the initially desialylated DPPIV was shifted back to the more acidic part of the gel, there comigrating with the sialylated form of the enzyme. This resialylated portion increased within the first hour of reculture and then reached a plateau (Fig. 11, *A* and *B*). As estimated by densitometry of the fluorographs, the resialylated form of DPPIV made up ~6% of total biotinylated cell surface DPPIV. No complete shift of the asialoform of DPPIV to the fully sialylated form was observed, indicating that DPPIV is only partly resialylated.

For TfR no resialylation could be detected. As shown in Fig. 11 *C* digestion of cells with neuraminidase after removal of Tf caused a shift of the receptor polypeptide bands to a more basic isoelectric point. This pattern of the asialoform of the receptor did not change during reculture for 11 h. Resialylated forms of TfR could not be detected even on overexposed gels. The same result was obtained, when ^{125}I-asialo-Tf was monitored for resialylation during reculture for up to 20 h. Compared to native Tf, asialo-Tf exhibited a clear shift in the IEF gel that did not change during reculture (Fig. 12). Hence, both TfR and Tf are not resialylated and do not return to the *trans*-Golgi or the TGN in detectable amounts in HepG2 cells.
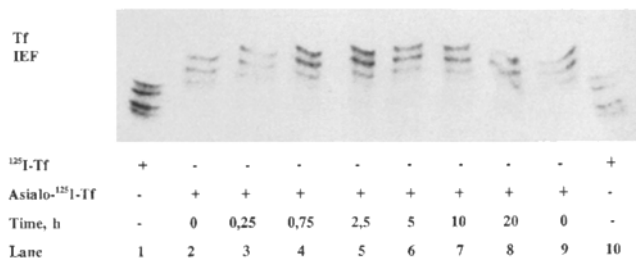
Discussion

In the present paper the different sites where recycling surface glycoproteins may reenter the secretory pathway were examined in HepG2 cells. Employing a novel experi-

Figure 11. Return of surface DPPIV (*A* and *B*) and TfR (*C*) to sites of sialyltransferases. HepG2 cells were labeled for 4 h with L-[^{35}S]methionine (5.5 MBq/3 × 10^6 cells), chased for 1 h, and were surface labeled with NHS-SS-biotin at 4°C. Cells were then treated with deferoxamine mesylate as described in Material and Methods, and were incubated for 2 h at 4°C with neuraminidase (200 mU/3 × 10^6 cells). After extensive washing and rewarming to 37°C cells were recultured for the times indicated. DPPIV and TfR were immunoadsorbed from detergent extracts, and the biotinylated forms of DPPIV and TfR were obtained by affinity chromatography on streptavidin agarose. Samples were separated by IEF (basic side at the top) and fluorographed. (*A*) DPPIV: Lane *1*: Nonbiotinylated cells as control for unspecific binding to streptavidin agarose. Lanes *3–6* and *9*: Biotin-labeled cells treated with neuraminidase. Lanes *2*, *7*, and *8*: Biotinylated cells treated likewise, but without neuraminidase treatment. (*B*) Kinetics of resialylation of surface DPPIV. The fraction of desialylated and of resialylated DPPIV was determined by densitometry of the fluorographs of (*A*). The fraction of resialylated DPPIV was calculated as the percentage of total cell surface DPPIV (desialylated plus resialylated) and was plotted versus time of reculture. (*C*) TfR: Lanes *2–5*: Biotin-labeled cells treated with neuraminidase. Lanes *1* and *6*: Biotin-labeled cells treated likewise, but without digestion with neuraminidase.

mental approach that combines covalent labeling of surface proteins with biotin (Busch et al., 1989; Loch et al., 1993) with modifications generated in the glycan moiety of the glycoproteins under study, membrane glycoproteins recycling from the cell surface to the Golgi apparatus and the TGN could be clearly distinguished from newly synthesized glycoproteins trafficking through these subcellular compartments en route to the surface. This allowed us to trace trafficking of membrane glycoproteins from the cell surface back to the Golgi apparatus and the TGN under utmost reliable conditions. Three major results were obtained. First, the data demonstrate that cell surface proteins may return not only to the TGN, but also to the *trans*-Golgi. Second, in contrast no detectable recycling to the *cis*-Golgi was noted indicating that earlier parts of the biosynthetic route are not accessible or are accessible at

^{125}I-Tf	+	-	-	-	-	-	-	-	-	+
Asialo-^{125}I-Tf	-	+	+	+	+	+	+	+	+	-
Time, h	-	0	0,25	0,75	2,5	5	10	20	0	-
Lane	1	2	3	4	5	6	7	8	9	10

Figure 12. Resialylation of asialo-Tf. Cells (3×10^6) were incubated in 4 ml DME containing 100 μM asialofetuin and 650 μM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid for 30 min. After addition of Fe^{3+}-loaded ^{125}I-asialo-Tf (1.5 μg, 4 kBq/μg) cells were further cultured for up to 20 h. At the times indicated ^{125}I-Tf in the culture medium was analyzed by isoelectric focusing as described in Material and Methods.

insignificant rates to recycling surface proteins. Third, surface TfR did not return to either one site of the biosynthetic route in detectable amounts despite its high rate of internalization. Accordingly, internalized TfR does not routinely enter pathways connecting endocytic to secretory routes in HepG2 cells pointing to the existence of an efficient sorting mechanism presumably in endosomes.

First, return to the *trans*-Golgi and to the TGN was examined monitoring resialylation of desialylated surface glycoproteins. Sialyltransferases transferring sialic acid residues to N-linked glycans have been shown to be localized in the TGN and the *trans*-Golgi, and are generally used as marker enzymes for the *trans*-Golgi/TGN (for reviews see Roth, 1987; Paulson and Colley, 1989; Broquet et al., 1991). Hence, resialylation of surface glycoproteins reports that these proteins reenter the biosynthetic route either at the *trans*-Golgi or at the TGN.

Resialylation could be clearly demonstrated for cell surface DPPIV. Examination of the time course showed that resialylation of DPPIV increased during the first hour of reculture and then reached a plateau phase. The most likely explanation for this plateau is that it represents the steady state between resialylation and simultaneous desialylation of cell surface DPPIV. Desialylation of cell surface DPPIV occurring during internalization/recycling has been shown recently (Kreisel et al., 1988). Using a different experimental approach Duncan and Kornfeld (1988) observed that resialylation of the cation-dependent and -independent mannose 6-phosphate receptor in CHO clone 13 cells reaches a similar plateau, and came to the same conclusion. The time-course of resialylation of DPPIV indicates that ~6% of total cell surface DPPIV are routed through the *trans*-Golgi or the TGN (Fig. 11, *A* and *B*). Since ~10% of surface DPPIV is internalized during this period, roughly estimated half of internalized DPPIV molecules may return to the biosynthetic route. It is noteworthy that resialylation of DPPIV was incomplete as suggested by the observation that the resialylated DPPIV polypeptides had a slightly more basic isoelectric point compared to the native sialylated DPPIV molecules. This could be explained by the assumption that the recycling asialo-forms of DPPIV encounter only a subset of sialyltransferases in that they preferentially recycle to the TGN, and not to the *trans*-Golgi. On the other hand sialyltransferases might act upon the asialoform of a recycling sur-

face glycoprotein in a different way as compared to newly synthesized glycoproteins. Our results demonstrating resialylation of DPPIV are in accordance with recent results obtained for the cation-dependent and -independent mannose 6-phosphate receptor, synaptophysin and the low density lipoprotein receptor and for several other surface proteins not yet characterized (Duncan and Kornfeld, 1988; Brändli and Simons, 1989; Green and Kelly, 1990, 1992; Huang and Snider, 1993). The results of these studies and of the present paper that were obtained in different cell lines using different experimental approaches clearly show that a restricted set of surface proteins return at least to the TGN.

Since resialylation of surface proteins can not discriminate between return to the TGN and the *trans*-Golgi, return to sites of fucosyltransferases was examined. Fucosyltransferases have been localized to the *medial* and to the *trans*-Golgi (for review see Roth, 1987). Hence, transfer of fucose residues to recycling surface glycoproteins reports return of these proteins at least to the *trans*-Golgi. Transfer of fucose residues to recycling surface glycoproteins could indeed be demonstrated for DPPIV, although to a low extent, showing that DPPIV does return not only to the TGN, but also to the *trans*-Golgi. Transfer of fucose residues could also be shown for a restricted set of surface glycoproteins, whereas most surface glycoproteins did not acquire fucose residues during recycling. Both, transfer of fucose and sialic acid residues by fucosyl- and sialyltransferases requires the presence of nucleotide sugar substrates GDP-fucose and CMP-sialic acid in addition to the glycosyltransferases. Intraluminal pools of nucleotide sugars were found in the Golgi where they are taken up from the cytoplasm by specific transport systems (Hirschberg and Snider, 1987; Milla et al., 1992). On the cell surface nucleotide sugars are not present in sufficient concentrations. In endosomes as a second potential extra Golgi site for reglycosylation, nucleotide sugars and nucleotide sugar carriers have so far not been described. Therefore, reglycosylation of recycling surface glycoproteins outside the Golgi apparatus or the TGN is highly unlikely.

Surprisingly, no resialylation and no transfer of fucose residues could be detected for the TfR during an experimental period of 11 h, i.e., during a period exceeding the half-life of the receptor in HepG2 cells (Figs. 9 *A* and 11 *C*). In addition, no resialylation could be detected for asialo-Tf even during 20 h of reculture when blocking ASGPR with asialofetuin (Fig. 12). According to these results TfR and the TfR/Tf complex do not return to the TGN at significant rates, but directly recycle from endosomes to the cell surface in HepG2 cells. Although a minimal degree of resialylation of TfR and of the TfR/Tf complex cannot be excluded within the inherent limitations of the methodology used, the fraction of surface TfR that recycles to resialylation sites would be extremely low, if not negligible in view of the high frequency of TfR internalization. It has been reported (Regoeczi et al., 1982, 1984) that asialo-Tf can be resialylated when injected into rats. This resialylation of asialo-Tf, however, is due to binding of asialo-Tf to the ASGPR and does not involve the TfR (Regoeczi and Koj, 1985). TfR of HepG2 cells seems to share commom features with lysosomal acid phosphatase in BHK cells that recycles 15–50 times in between the

plasma membrane and endosomes before being routed to lysosomes, but does not return to the *trans*-Golgi/TGN (Braun et al., 1989). Both TfR and lysosomal acid phosphatase are internalized via coated pits and coated vesicles. In addition, several other surface proteins (Brändli and Simons, 1989), including the GPI-anchored 5'-nucleotidase (van den Bosch et al., 1988), and the H2-K antigen (Reichner et al., 1988) were shown not to return to the TGN. On the other hand, TfR has been shown to be resialylated in K562 erythroleukemia cells and EL-4 murine T-cell lymphoma cells (Snider and Rogers, 1985; Reichner et al., 1988) pointing to cell type-related differences. The finding that TfR is neither refucosylated nor resialylated in HepG2 cells although this glycoprotein routinely enters the endosomal compartment during endocytosis/recycling further supports the conclusion that reglycosylation of recycling surface glycoproteins does not take place in endosomes.

The question arises why some proteins from the cell surface are recycled to the *trans*-Golgi and to the TGN, but most of them are not. Stoorvogel et al. (1988) developed a model in that early endosomes communicate predominantly with the plasma membrane, while late endosomes interact preferentially with the TGN. They described TfR as a protein that exits very early from the endocytic pathway in HepG2 cells, being separated from the mannose 6-phosphate receptor even when both receptors were internalized together (Stoorvogel et al., 1989). This model was further supported by Dunn et al. (1989) showing that low density lipoprotein receptor accumulates in early endosomes while TfR is permanently extracted from this compartment to recycling endosomes. Those receptors that are incompletely retrieved in the early endosomes are further transported to the late endosomes, from where they may be routed to the TGN. Vesicular transport between endosomes and the TGN has been shown in vitro (Goda and Pfeffer, 1988), and is supported by the observation that brefeldin A induces the fusion of these two compartments (Lippincott-Schwartz et al., 1991; Wood et al., 1991). Moreover, it has been shown that transport between late endosomes and the TGN is stimulated by rab9, a member of the rab family serving as key regulators of vesicular transport (Lombardi et al., 1993; for review see Rothman and Orci, 1992). On the other hand, rab4 and rab5 are localized in early endosomes and in the plasma membrane and mediate fusion between endosomes and the cell surface membrane (Bucci et al., 1992; van der Sluijs et al., 1992). The fact that DPPIV, but not TfR may return from the cell surface to the *trans*-Golgi and the TGN in HepG2 cells is likely due to a different sorting efficiency of both proteins in early endosomes. Whereas TfR is almost completely extracted from early endosomes and directly routed to the cell surface, DPPIV at least partly reaches late endosomes in HepG2 cells. In K562 erythroleukemia cells and EL-4 murine T-cell lymphoma cells where TfR may be resialylated, sorting of TfR in early endosomes is obviously not as efficient as in HepG2 cells (Snider and Rogers, 1985; Reichner et al., 1988), indicating that modes of membrane trafficking at least quantitatively differ between cell types. Based on the observation that cell types specialized for protein secretion exhibit more endocytic traffic directed to the TGN than do nonsecretory cell types, it has been suggested that these differences between cells may reflect the variant requirement to transport secretory vesicle membranes from the cell surface to the Golgi apparatus (Green and Kelly, 1990).

No recycling of surface DPPIV and TfR to the sites of mannosidase I could be detected in HepG2 cells over a period of 12 h (Fig. 6). Control experiments clearly showed that biotinylated cell surface DPPIV can be trimmed by Golgi mannosidases in principle and that, hence, the inability to demonstrate return to Golgi mannosidases is not due to a resistance of cell surface DPPIV to these enzymes. Moreover, it was proven that after inhibition of mannosidase I by dMM enzyme activity was fully restored after washout of the inhibitor, as has also been demonstrated by other groups (Snider and Rogers, 1985; Duncan and Kornfeld, 1988; Neefjes et al., 1988), ruling out that our negative results reflect a persisting inactivation of this enzyme. Since Mannosidase I has been localized to the *cis*-Golgi in a variety of cell types (for review see Roth, 1987), it is concluded that DPPIV and TfR do not significantly return to early Golgi compartments. Although reprocessed forms of DPPIV and TfR could not be detected even after overexposure of the gels shown in Fig. 6, it cannot be excluded that minute fractions of these glycoproteins recycling to the *cis*-Golgi might escape detection. However, with respect of the sensitivity of the method employed and having in mind that recycling was monitored for as long as 12 h, such a fraction would not represent a significant recycling pathway. Very recently, mannosidase I has also been detected in middle and even partly in *trans*-Golgi elements of rat kidney cells, pancreatic acinar cells, enterocytes, goblet cells and hepatocytes (Velasco et al., 1993). The results of the present paper demonstrating transfer of fucose residues to recycling surface proteins, but no reprocessing by mannosidase I, suggest that fucosyltransferases and mannosidase I are not colocalized in Golgi elements encountered by surface proteins during recycling. Our results are in contrast to those of Snider and Rogers (1986) obtained by example of TfR in K562 cells that surface proteins may routinely return to the *cis*-Golgi and that most of the glycoprotein traffic through the Golgi complex is composed of recycling surface proteins. Our results are in agreement with that of Neefjes et al. (1988) and Duncan and Kornfeld (1988), who could not detect significant transport of TfR and of the cation-dependent and -independent mannose 6-phosphate receptor, respectively, from the cell surface to the site of mannosidase I. In none of these three studies, however, recycling of surface glycoproteins to the *cis*-Golgi was examined by example of membrane glycoproteins that were covalently labeled on the cell surface beforehand. Hence, contamination of recycling surface glycoproteins by newly synthesized glycoproteins could not be excluded unequivocally. In comparison, the experimental system used in the present study clearly allows to conclude that recycling of surface TfR and DPPIV through the *cis*-Golgi is insignificant.

As compared to other methods employed in studies on recycling pathways such as immunolocalization and subcellular fractionation, the approach used in this study measures oligosaccharide modifications effected on surface glycoproteins by oligosaccharide processing enzymes during recycling through the various Golgi compartments.

Recycling of those surface glycoproteins that are processed by these enzymes to a low extent might, therefore, be underestimated. Within the limits of resolution of this experimental system, the results of the present paper comparing return of surface proteins to three different sites of the biosynthetic route suggest that the compartments of the biosynthetic pathway in HepG2 cells are divided into those that are accessible to surface proteins (TGN, *trans*-Golgi) and those that are not accessible at significant rates to recycling proteins (*medial/cis*-Golgi). Return to the TGN/*trans*-Golgi is restricted to distinct surface proteins, whereas others directly recycle from early endosomes to the cell surface. Most likely, proteins like TfR that do not return significantly to the secretory pathway are efficiently extracted from early endosomes and recycled back to the plasma membrane, whereas proteins like DPPIV that may reenter the secretory route are transported to late endosomes, from where they may be routed to the TGN. Except the intracellular recycling of the mannose 6-phosphate receptor from late endosomes to the TGN (for review see Kornfeld and Mellman, 1989), the function of membrane protein traffic connecting the endocytic to the biosynthetic pathway remains to be characterized. It has been postulated that recycling via the Golgi apparatus or the TGN may serve to remove internalized membrane proteins from the lysosomal pathway (Green and Kelly, 1992). Taking into account that cell surface glycoproteins continuously lose terminal sugar residues (Tauber et al., 1983, 1989; Kreisel et al., 1988), resialylation and refucosylation during recycling via the *trans*-Golgi and the TGN might also serve as a repair mechanism for truncated oligosaccharides on surface glycoproteins. Moreover, the mechanisms that cause the different intracellular routing of recycling TfR and DPPIV are still not clear. Different routing during recycling might be regulated by sorting signals in the cytoplasmic domain of the proteins. On the other hand, the observation that modes of membrane trafficking may differ considerably between cell types indicates that the routes of recycling are not only defined by specific signals on intrinsic membrane proteins, but are also subject to other regulatory mechanisms.

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