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 biosynthetic compartments from the cell surface and newly synthesized glycoproteins that pass these compartments during biosynthesis en route to the surface. Return 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.

During internalization and recycling plasma membrane glycoproteins of different function may return from the cell surface to the trans-Golgi network (TGN) 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

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537
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 in part studied by means of proteins that had not been labeled on the cell surface, or had been labeled non-covalently 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 technical route 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 about 5 min at the cell surface (Ciechanover et al., 1983), and the serine peptidase DPP IV (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, DPP IV, 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 (Eugensteig, Germany); other materials for cell culture were purchased from Falcon (Heidelberg, Germany) or Nunc (Wiesbaden, Germany). L-[35S]Met/hione (>48 TBq/mmol), L-[6-3H]fucose (>800 GBq/mmol), D-[6-3H]galactose (0.74 TBq/mmol), D-[2-3H]mannose (370 GBq/mmol), [35S]sodium iodide (3.7 GBq/ml) and UDP-D-[14C]galactose (7.4 GBq/mmol) were from Amersham Buchler (Braunschweig, Germany). BSA, NP-40, streptavidin agarose, deferoxamine mesylate, n-N-dithiotreitol, and iodoacetamide were purchased from Sigma (Deisenhofen, Germany). Sulfosuccinimidyl-2-(biotiamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin), sulfosuccinimidyl-6-(biotiamido)hexanoate, and IodoGen iodination reagent (1,3,4,6-tetrachloro 3a-6a-diphenylglycoluril) were obtained from Pierce (Oude Beijerland, The Netherlands). Protein A-Sepharose and amphotilines (pH 3.5–5.9, 45%) were from Pharmacia (Freiburg, Germany). Endo-β-N-acetylglucosaminidase H (Endo H) from Streptomyces pilatus (EC 3.2.2.19), peptide N4-(N-acetyl-β-glucosaminy1) asparagine amidase F (PNGase F) from Flavobacterium meningoepiticum (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 Manα,α-GlcNAc utilized as standards were from Oxford Glycosystems (Abingdon, UK). Unless otherwise stated, all other chemicals and regents 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% CO2 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 TIR 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 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-[35S]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-[35S]methionine (5.5 MBq/3 × 10⁶ 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 D-[6-3H]fucose (15 MBq/3 × 10⁶ cells), D-[2-3H]mannose (18 MBq/3 × 10⁶ cells), or D-[6-3H]glactose (7.4 MBq/3 × 10⁶ 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/Ca2⁺/Mg2⁺ for 20 min on a rocking platform at 4°C. Cells were then washed with PBS/Ca2⁺/Mg2⁺ (8 mM Na2HPO4, 1 mM KH2PO4, pH 7.2), 3 mM KCl, 137 mM NaCl, 0.9 mM CaCl2, 0.5 mM MgCl2), and incubated with a freshly prepared solution (1 mg/ml) of NHS-SS-biotin in PBS/Ca2⁺/Mg2⁺ for 20 min on a rocking platform at 4°C. Cells were then washed twice with PBS/Ca2⁺/Mg2⁺ containing 0.1% (mass/vol) BSA, twice with PBS/Ca2⁺/Mg2⁺, 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 mM EDTA, 10 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, 2500 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).

Immunofluorescence Absorption

All steps were carried out at 4°C. Cells were washed four times with ice-cold PBS/Ca2⁺/Mg2⁺, 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 mM/10⁶ 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 glass 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 immunofluorescence absorption 10 μg of monoclonal anti-TIR 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. No adsorption was observed. Immunocomplexes were collected by binding to protein A-Sepharose CL-4B precoated with affinity-purified rabbit anti-mouse IgG. For precocating, 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 2 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 50 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 TIR, 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 of 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, 10 μg protein A-Sepharose with the immunoadsorption mixture, washing of the beads and elution of immunocomplexes was done as described for TIR, except that SDS was used at a concentration of 0.1% instead of 0.5% in the last washing buffer.

SDS–Polyacylamide Gel Electrophoresis

Proteins were separated on 7.5% SDS–polyacrylamide slab gels (Laemmli, 1970) and stained with silver (Heukeshoven and Dernick, 1988). Gels with 7.5% or 10% [H]-labeled samples were processed and fluorographed according to Bonser and Laskey (1974) using Kodak XAR-5 x-ray film. Bands on fluorograms were quantitated by densitometry using a Hirschmann Elsco 400 Scanner (Hirschmann, München, Germany). Molecular mass standards were α₂-macroglobulin from human plasma (180 kD), β-galactosidase from Escherichia coli (116 kD), fructose-6-phosphatase from rabbit muscle (84 kD), pyruvate 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. TIR and DPPIV bound to streptavidin agarose were eluted with 25 μl of 50 mM 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] amphotolines (40%), pH 3.5–9.5), 97 mM dithiothreitol. Vertical 4% polyacrylamide gels containing 9.0 M urea, 2% (by vol) NP-40, 6% of a mixture of 40% amphotolines (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 H3PO4 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 Na2dithiorthreitol, 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 105°C in 1 N HCl. The resulting samples were analyzed by HPLC according to Lohmander (1986) on a HPLC (300 mm × 7.8 mm; Bio-Rad, München, Germany) using 0.1 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-Acetylglucosaminic acid, L-fucose, β-mannose, β-galactose, N-acetyl-β-galactosamine and N-acetyl-α-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% sucrose, 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 Mc-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, chymotain, and pepstatin [10 μg each]) was added after cooling. Digestion with 10 U of Endo H was performed in 20 mM sodium phosphate buffer, pH 5.5, 0.1% (by vol) PNGase F, and that with 10 U of PNGase F in 20 mM sodium phosphate buffer, pH 7.5, 0.1% (by vol) NP-40, and that with 10 U neuraminidase from Vibrio cholerae in 20 mM sodium phosphate, pH 5.5, 4 mM CaCl2.

Treatment of Cells with Neuraminidase
Cells (3 × 106 in 100 mm-dishes) were cooled on ice, washed three times with ice-cold PBS, pH 5.5, 1 mM CaCl2, 1 mM MgCl2, and were then incubated with 200 μM of neuraminidase (from Clostridium perfringens) in 4 ml PBS, pH 5.5, 4 mM CaCl2, 1 mM MgCl2, for 2 h at 4°C on a gently rocking platform. After washing five times with PBS/Ca2+/Mg2+, 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 biotinylated at the surface at 4°C, treated with deferoxamine mesylate (0.4 mM in PBS, pH 5.5, 1 mM CaCl2, 1 mM MgCl2, 0.05 mM deferoxamine mesylate to dissociate Tf from cell surface TfR, and treated with neuraminidase. After digestion for 2 h, the cultures were centrifuged, washed twice with PBS/Ca2+/Mg2+, and detergent extracted. For analysis of recycling to the TGN, cells (3 × 106 in 100-mm dishes) were preincubated for 30 min with 4 ml of DME containing 100 μM atosilofetuin, 650 μM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid. Incubation was continued after addition of Fe3+-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. Fe3+-Loaded asialo-Tf was prepared in that Tf was loaded with Fe3+ 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 μM neuraminidase (Clostridium perfringens) immobilized to agarose (Sigma) and suspended in 200 μl PBS, pH 5.5, 4 mM CaCl2, containing antipain, pepstatin, chymotain, and leupeptin at a final concentration of 20 μg/ml each.

Analysis of Recycling to the trans-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 × 106 cells) was added, and the incubation was continued for 6 or 12 h, all in the presence of dMM. Cells were then washed four times with ice-cold PBS/Ca2+/Mg2+, and detergent extracted. For analysis of incorporation of L-[6-3H]Fucose binding to 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

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 × 106 cells) was added, and the incubation was continued for 6 or 12 h, all in the presence of dMM. Cells were then washed four times with ice-cold PBS/Ca2+/Mg2+, and detergent extracted. For analysis of incorporation of L-[6-3H]Fucose binding to 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...
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, following 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 n-[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 5 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 immunoadsorption and analyzed by 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 3 ml methanol, 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 Pfieffer 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 Man₅GlcNAc² isolated from thyroglobulin and from ribonuclease A (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 mannosse 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 mannosse 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 1-[6-3H]fucose, n-[2-3H]mannose and n-[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 of both 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.
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-[35S]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 by 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-[35S]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-[35S]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
were detergent extracted. For determination of half-lives TfR were cooled to 4°C, surface-labeled with NHS-SS-biotin, and de-
tergent extracted. Surface TfR in HepG2 cells. Surface TfR in nonbiotinylated cells (protocol b): Cells were pulse labeled with L-[35S]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 fluoro-
graphed. 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-[35S]methionine (5.5 MBq/3 × 10⁶ 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-[35S]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 (t₁/₂) were calculated from the slope of the regression line, and correlation coefficients (r) were calculated. Half-lives are the means of three independent experiments.

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 fluoro-
graphed. The fluorograms were densitometered, and the percentage of internalization of TfR and DPPIV was calculated as detailed in Material and Methods.

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 7) 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 absence of the inhibitor (lanes 1 and 7) in comparison to a molecular mass of 125 and 95 kD, respectively, observed in the presence of dMM (lanes 3 and 8). Likewise, both gly-
coproteins 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 label-
ing. 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-[35S]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-[<sup>35</sup>S]methionine (5.5 MBq/3 × 10⁶ 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-[<sup>35</sup>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 of the cells. Biotinylated forms (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 DPPIV was incubated in vitro with isolated Golgi apparatus and was, thereafter, analyzed with respect to trimming of its oligomannosidic oligosaccharides. Cells were metabolically labeled with D-[<sup>2</sup>-<sup>3</sup>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. Biotinyl-

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 × 10⁶ cells) with L-[<sup>35</sup>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.
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 Man9GlcNAc2 and Man9GlcNAc2 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 Man8_9GlcNAc2 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 Man9GlcNAc2 with Man9GlcNAc2 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 lose 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 reflect 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 non-processed 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.
whereas incorporation of \[^{3}H\]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 \[^{3}H\]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 \[^{3}H\]fucose into biotinylated TfR was detectable even after 60 min of exposure of the film (Fig. 9 A, lane 6). On the other hand, newly synthesized TfR was labeled with \[^{3}H\]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 non-biotinylated control cells (Fig. 9 A, lane 5). Moreover, as expected, the newly synthesized form of DPPIV generated during 12 h of reculture exclusively had an apparent molecular mass of 110 kD (Fig. 9 A, lanes 3 and 4).

Incorporation of \[^{3}H\]fucose into surface glycoproteins was not restricted to DPPIV. Analysis of the whole set of biotinylated proteins after reculture of cells with \[^{3}H\]fucose for 6 h or 12 h, demonstrated that \[^{3}H\]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, \[^{3}H\]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 \[^{3}S\]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 siayltransferases.

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 \( \times \) 10^6 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
Desialylation of surface TfR with neuraminidase.

HepG2 cells were labeled for 4 h with L-[35S]methionine (5.5 MBq/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 x 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 x 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 x 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 DPPIV 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.

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 125I-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 experimental 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 to detectable recycling to the cis-Golgi was noted indicating that earlier parts of the biosynthetic route are not accessible or are accessible at
Resialylation of asialo-Tf. Cells (3 x 10⁶) 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 Fe³⁺-loaded ¹²⁵I-asialo-Tf (1.5 µg, 4 kBq/µg) cells were further cultured for up to 20 h. At the times indicated ¹²⁵I-Tf in the culture medium was analyzed by isoelectric focusing as described in Material and Methods.

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 mannos 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 sialyltransf-
plasma membrane and endosomes before being routed to lysosomes, but does not return to the trans-Golgi/TGN (Braun et al., 1989). Both TIR and lysosomal acid phosphatase are internalized via coated pits and coated vesicles. In addition, several other surface proteins (Brandli 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, TIR 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 TIR is neither refucosylated nor resialylated in HepG2 cells although this glycoprotein routinely enters lysosomes, but does not return to the plasma membrane and mediate fusion between endosomes and the plasma membrane glycoproteins that were covalently labeled on the cell surface membrane (Bucci et al., 1992; van der Sluijs et al., 1992). The fact that DPPIV, but not TIR 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 TIR 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 TIR may be resialylated, sorting of TIR 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 TIR 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 TIR do not significantly return to early Golgi compartments. Although reprocessed forms of DPP IV and TIR 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 TIR 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 TIR and of the cation-dependent and -independent mannoside 6-phosphatase 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 TIR and DPP IV 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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