The Pathways of Endocytosed Transferrin and Secretory Protein are Connected in the *trans*-Golgi Reticulum

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Abstract. We used a conjugate of transferrin and horseradish peroxidase (Tf/HRP) to label the intracellular transferrin receptor route in the human hepatoma cell line HepG2. The recycling kinetics of [^{125}I]Tf/HRP were similar to those of unmodified [^{125}I]Tf, implying identical routes for both ligands. 3,3'Diaminobenzidine (DAB)-cytochemistry was performed on post-nuclear supernatants of homogenates of cells which were incubated with both Tf/HRP and [^{125}I]Tf, and caused two different effects: (*a*) the equilibrium density of [^{125}I]Tf containing microsomes in a Percoll density gradient was increased, and (*b*) the amount of immunoprecipitable [^{125}I]Tf from density-shifted lysed microsomes was only 20% of that of nonDAB treated microsomes. The whole biosynthetic route of α_1-antitrypsin (AT), a typical secretory glycoprotein in HepG2 cells, was labeled during a 60-min incubation with [^{35}S]methionine. DAB cytochemistry was performed on post-nuclear supernatants of homogenates of cells which were also incubated with Tf/HRP. DAB cytochemistry caused ~40% of microsome-associated "complex" glycosylated [^{35}S]α_1-antitrypsin ([^{35}S]c-AT) to shift in a Percoll density gradient. Only part of the density shifted [^{35}S]c-AT could be recovered by immunoprecipitation. A maximum effect was measured already after 10 min of Tf/HRP uptake. The density distribution of the "high mannose" glycosylated form of ^{35}S-α_1-anti-trypsin ([^{35}S]hm-AT) was not affected by Tf/HRP. If in addition to Tf/HRP also an excess of non-conjugated transferrin was present in the medium, [^{35}S]c-AT was not accessible for Tf/HRP, showing the involvement of the transferrin receptor (TfR) in the process. Furthermore, we show that if Tf/HRP and [^{35}S]c-AT were located in different vesicles, the density distribution of [^{35}S]c-AT was not affected by DAB-cytochemistry. Pulse-labeling with [^{35}S]methionine was used to show that [^{35}S]c-AT became accessible to endocytosed Tf/HRP minutes after acquirement of the complex configuration. A common intracellular localization of endocytosed Tf/HRP and secretory protein could be confirmed by immuno-electron microscopy: cryosections labeled with anti-albumin (protein A-colloidal gold) as well as DAB reaction product showed double-labeling in the *trans*-Golgi reticulum.

ENDOCYTOSIS causes a considerable membrane flow from the plasma membrane to intracellular compartments. Exocytosis of newly synthesized secretory and membrane proteins on the other hand generates a membrane flow in the opposite direction. To maintain stable organelle membrane quantities, both processes must be in balance. Whether, and how these two membrane routings are correlated, has recently been the objective of many studies. The Golgi complex and particularly the *trans*-Golgi reticulum (TGR) seem to play an important role in membrane dynamics (for reviews see Farquhar, 1985; Griffiths and Simons, 1986).

In hepatoma cells, exocytotic proteins are transported with characteristic kinetics between the rough ER and the Golgi complex, while the transport rate between the Golgi complex and the plasma membrane is uniform (Strous and Lodish, 1980; Lodish et al., 1983). The formation of "complex" out of "high mannose" glycosylated proteins occurs during the second stage of transport (reviewed by Kornfeld and Kornfeld, 1985). the TGR is part of the post Golgi secretory route; exocytotic albumin and Vesicular Stomatitis virus glycoprotein were co-localized in this compartment (Strous et al., 1983; Zijderhand-Bleekemolen et al., 1987).

During endocytosis, many receptors shuttle between endosomal compartments and the plasma membrane. A well-studied example is the transferrin receptor. It mediates the endocytosis of transferrin (Tf), which releases its iron intracellularly at acidic pH and recycles receptor-bound to the plasma membrane (Klausner et al., 1983; Dautry-Varsat et al., 1983). The intracellular transport occurs through compartments with a transient character (Ajioka and Kaplan, 1986). Although the recycling kinetics of the Tf-TfR complex are well known (Ciechanover et al., 1983*a*, *b*), the precise

1. *Abbreviations used in this paper*: AT, α_1-anti-trypsin; c-AT, "complex" glycosylated α_1-anti-trypsin; hm-AT, "high mannose" glycosylated α_1-anti-trypsin; DAB, 3,3' diaminobenzidine; HRP, horseradish peroxidase; MEM, Eagle's Minimal Essential Medium; SPDP, N-succinimidyl-3-2-pyridyldithiopropionate; Tf, transferrin; Tf/HRP, transferrin/horseradish peroxidase conjugate; TfR, transferrin-receptor; TGR, *trans*Golgi reticulum.

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recycling pathway has not yet been elucidated. Many studies argue in favor of the involvement of the Golgi cisternae and the TGR in TfR recycling. Morphologically, a significant TfR pool has been localized in the TGR (Willingham and Pastan, 1985). After endocytosis, plasma membrane TfR (Woods et al., 1986) and extracellularly added Tf (Hedman et al., 1987) have been detected in the Golgi complex and TGR respectively, showing the accessibility of these compartments for endocytosed Tf-TfR. Biochemical data also support the idea of TfR recycling through post-Golgi compartments. Both, desialylated TfR (Snider and Rogers, 1985), and endocytosed asialotransferrin (Regoeczi et al., 1982) were resialylated during endocytosis, indicating passage through sialyltransferase containing Golgi cisternae or TGR (Roth et al., 1985). If TfRs recycle through Golgi or post-Golgi compartments they should meet exocytotic proteins. Indeed, endocytosed Tf has recently been detected in choline-esterase containing Golgi-derived coated vesicles (Fishman and Fine, 1987), and in vesicular stomatitis virus glycoprotein containing Golgi-associated structures (TGR) (Hedman et al., 1987). As outlined above, it is not clear whether other compartments of the biosynthetic route are involved in Tf-TfR recycling, and also not where the exact merging site of both routes is located.

In this study we have used Tf/HRP to label all intracellular compartments involved in TfR-ligand recycling with peroxidase activity. Using DAB cytochemistry (Courtoy et al., 1984; Stoorvogel et al., 1987) we show that in HepG2 cells newly synthesized AT, reached Tf/HRP containing compartments within minutes after its complex glycosylation. Morphologically we show the occasional concomitant presence of Tf/HRP and secretory albumin in vesicles located at the *trans*-Golgi region. These findings not only show that at least part of the endocytosed TfR-ligand merges with the exocytotic route but also that this takes place in the TGR.

Materials and Methods

Materials

The human hepatoma cell line HepG2, clone A 16 (Schwartz et al., 1983) was cultured as described earlier (Stoorvogel et al., 1987). Transferrin and HRP (type VI) were obtained from Sigma Chemical Co. (St. Louis, MO). N-succimidyl-3-2-pyridyldithio propionate (SPDP), Sephadex G25, Sephacryl S 200, Percoll, and Percoll density marker beads were purchased from Pharmacia (Uppsala Sweden), Centricon 10 microconcentrator filters from Amicon, [^{35}S]methionine from Amersham (The radiochemical center, England), and rabbit anti-human α_1-antitrypsin from DAKO PATTS (Denmark). Rabbit anti-human transferrin was prepared in our laboratory.

Preparation of the Tf/HRP Conjugate

Apotransferrin was coupled to HRP by using SPDP as coupling reagent, principally as described by the manufacturer. In short, 10 mg Tf and 20 mg HRP were both incubated for 30 min at 20°C in 1 ml PBS containing 280 µM SPDP and 7.6 mM SPDP respectively. Tf and HRP were separated from noncoupled SPDP on Sephadex G 25 columns equilibrated in PBS and 0.1 M NaCl, 0.1 M acetate, pH 4.5, respectively. The HRP solution was concentrated to 1 ml using a Centricon microconcentrator. DTT was added up to 50 mM. After incubation for 20 min the HRP solution was filtrated on Sephadex G 25 in PBS. The Tf and HRP preparations were pooled, concentrated to 1 ml using a Centricon microconcentrator, and allowed to couple for 16 h at 4°C. The conjugates were separated from free HRP and Tf on a Sephacryl S-200 column in 150 mM NaCl, 20 mM Tris-HCl, pH 7.2. Fractions analyzed by SDS-PAGE, containing low molecular weight Tf/HRP conjugates were pooled. Then the conjugate was Fe^{3+} saturated similar to the procedure described for Tf (Stoorvogel et al., 1987), dialyzed and stored at -70°C in 150 mM NaCl, 20 mM Hepes/NaOH, pH 7.2. Tf was iron saturated after conjugation, to assure that only iron saturated conjugates, which were not blocked in their capacity of iron release, would bind the TfR. About 80% of the conjugation products in the pooled fractions were composed out of one molecule Tf and one molecule HRP. The amount of free Tf was less than 2%, and free HRP was not detectable. The Tf/HRP concentration was measured according to Bradford (1976), using Tf as a standard. The HRP activity was measured according to the method described by the manufacturer, and was not significantly affected by the conjugation procedure.

Ligand Iodination

Iron saturated Tf/HRP, and Tf were iodinated in 20 mM Hepes/NaOH, pH 7.2 using the method described before (Stoorvogel et al., 1987). The specific activities of both ligands were between 2-3 × 10^5 cpm/µg.

DAB Cytochemistry and Percoll Gradient Fractionation

DAB cytochemistry and Percoll gradient fractionation were generally as reported before (Stoorvogel et al., 1987). Instead of 2 mM $CaCl_2$, 1 mM EDTA was added to both the homogenization buffer, and the Percoll solution. This resulted in a reduced separation of plasma membranes and endosomes, but also reduced the loss of [^{125}I]transferrin and [^{35}S]AT in the nuclear pellet (not shown). Samples of 500 µl of the post nuclear supernatant were layered on top of 13 ml 20% Percoll in 0.25 M sucrose, 1 mM EDTA, 10 mM Hepes/NaOH, pH 7.2. Density gradients were formed during centrifugation at 39,900 g_{max} for 60 min at 4°C. The density distribution was measured with density marker beads.

Labeling of Cells with [^{35}S]Methionine, Tf/HRP, and [^{125}I]Tf

Semiconfluent cell cultures were washed three times and incubated for 2 h at 37°C in a 5% CO_2 atmosphere on a rocking platform in serum- and methionine-free culture medium, to deplete the cells of both serum Tf and non-labeled methionine. Then fresh medium containing 50-100 µCi/ml [^{35}S]methionine was added. In pulse/chase experiments, the medium was replaced after 10 min by fresh pre-equilibrated MEM containing 100 µM methionine. Receptor labeling was done by adding 5 µg/ml [^{125}I]Tf and 25 µg/ml Tf/HRP. The incubations were stopped by washing the cells at 0°C with MEM, containing 20 mM Hepes/NaOH, pH 7.2, and lacking bicarbonate (MEMH) (3 times quick, and 1 × 60 min). If indicated surface receptor bound ligand was removed by additional washing at 0°C for 10 min at pH 4.5, and 10 min at pH 7.2 as previously described (Stoorvogel et al., 1987). [^{35}S]AT was quantitatively immunoprecipitated from culture media, cell lysates and gradient fractions using the method described (Strous et al., 1982; Strous et al., 1985), and analyzed by SDS-PAGE. Percoll did not influence the immunoprecipitation. The gels were fluorographed and the fluorograms were scanned with a microdensitometer (E. C. apparatus, St. Petersburg, FL) for quantitation.

Electron Microscopy

Cells were subsequently incubated for 2 h in serum-free medium and for 30 min in medium containing 30 µg/ml Tf/HRP. The culture plates were washed three times with MEMH, and fixed in 0.1 M cacodylate, pH 7.4 containing 0.5% glutaraldehyde for 1 h at 0°C. The cells were washed with 0.1 M cacodylate buffer, pH 6.9 and incubated in buffer containing 1 mg/ml DAB and 1 mg/ml DAB, 0.01% H_2O_2 for 30 and 60 min respectively at 0°C. The cells were scraped from the dish and prepared for cryosectioning as previously described (Geuze et al., 1981). Cryosections were prepared according to Tokuyasu and Singer (1976). Immunolabeling of sections (Geuze et al., 1981) was done using affinity purified rabbit anti-human albumin (Zijderhand-Bleekemolen et al., 1987) and 9-nm protein A-gold complexes. The sections were stained at 0°C during a 10 min incubation in 2% methylcellulose containing 0.2% OsO_4.

Results

Binding of [^{125}I]Tf/HRP to Cells

Tf/HRP was used as a ligand for the TfR. The preparation method used yielded only low molecular weight conjugates

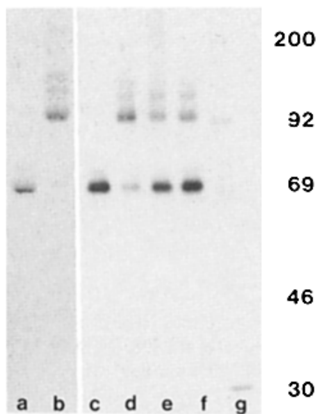

Figure 1. Analysis of Tf/HRP by PAGE. Samples of Tf (*a*), Tf/HRP (*b*), [125I]Tf (*c*), and [125I]Tf/HRP (*d*) were visualized after SDS-PAGE at non reducing conditions by Coomassie blue staining (*a* and *b*) or autoradiography (*c* and *d*). Tissue culture dishes (6-cm) were incubated for 2 h in MEMH to deplete the cells for exogenous transferrin. The cells were incubated for 60 min at 0°C in MEMH containing 5 μg/ml [125I]Tf/HRP. Excess ligand was washed away. Then receptor bound ligand was released at 0°C, by using the acidic washing procedure described in the experimental procedures section (*e*) or by incubation at 37°C for 30 min in MEMH (*f*). Ligand was recovered from the release media by immunoprecipitation, using rabbit antiserum against human Tf. The mass of molecular weight markers (*g*) is given in kilodaltons on the right.

which were practically free of non-conjugated Tf (Fig. 1, lane *b*). Iodination of Tf/HRP caused some hydrolysis of the conjugate, yielding both [125]Tf/HRP and [125I]Tf (Fig. 1, lane *d*). Binding at the cell surface at 0°C showed that [125I]Tf has a slightly higher affinity for the transferrin receptor than [125I]Tf/HRP (Fig. 1, lane *e*). Surface bound ligand, removed at 0°C, consisted of the same relative amounts of [125I]Tf and [125I]Tf/HRP as pre-bound ligand, released during endocytosis at 37°C (Fig. 1, lanes *e* and *f*). In both cases ~50% of the total radioactivity represented nonconjugated [125I]Tf. Therefore, recycling of [125I]Tf/HRP was as efficient as that of [125I]Tf. Furthermore, this shows that during recycling HRP remained associated with transferrin. In addition, we compared the rate of endocytosis and release at 37°C of prebound [125I]Tf/HRP and [125I]Tf (Fig. 2). Because the batch of [125I]Tf/HRP contained also some [125I]Tf, Fig. 2 *b* reflects the release of both [125I]Tf and [125I]Tf/HRP. The almost identical curves showing the release of [125I]Tf in one case (Fig. 2 *a*), and of [125I]Tf/HRP and [125I]Tf in the other, strongly suggests similar recycling rates for both ligand types.

To determine the binding specificity, HepG2 cultures were incubated for 30 min at 37°C, in the presence of various concentrations of [125I]Tf/HRP (Fig. 3). At low ligand concentrations, binding was undervalued, as a result of depletion of iron saturated ligand from the medium during the incubation period. Saturation of binding was obtained at ~15 μg/ml. Because the mixture of [125I]Tf and [125I]Tf/HRP showed saturation binding, it must be concluded that the binding of [125I]Tf/HRP is also saturable. Nonspecific binding of [125I]Tf/HRP, measured in the presence of excess non-labeled Tf (1 mg/ml), was less than 20% of total binding at 30 μg/ml. The total amount of [125I]Tf–binding sites was ~1.5×10^6 per cell (not shown).

Tf/HRP Mediated Density Shift of Endocytosed [125I]Tf

Cells were incubated for 30 min at 0°C or at 37°C in medium containing both 25 μg/ml pure Tf/HRP and 5 μg/ml [125I]Tf. After labeling the cells were homogenized and fractionated on a Percoll density gradient. If the cells were labeled on ice, a single peak of [125I]Tf–binding plasma membranes with a mean density of ~1.032 g/ml was observed (Fig. 4 *B*). An acidic wash or proteinase K treatment of the cells at 0°C before homogenization removed almost all

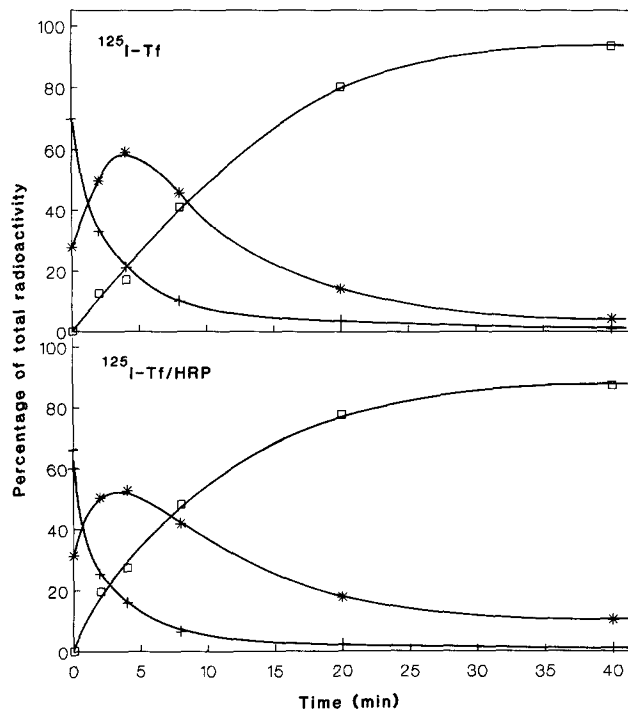

Figure 2. Uptake and release of pre-bound [125I]Tf and [125I]Tf/HRP. Tissue culture dishes (35-mm) were pre-treated as in Fig. 1, and incubated for 60 min at 0°C in MEMH containing 5 μg/ml [125I]Tf or [125I]Tf/HRP to label surface receptors. Nonbound ligand was washed away, and the cells were incubated at 37°C in MEMH for the times indicated. Ligand released in the incubation medium (□), surface-bound ligand sensitive to the acidic washing procedure described in the experimental procedures section (+), and ligand resistant to this procedure collected by dissolving cells in 1 N NaOH (*), were quantified in a gamma-counter.

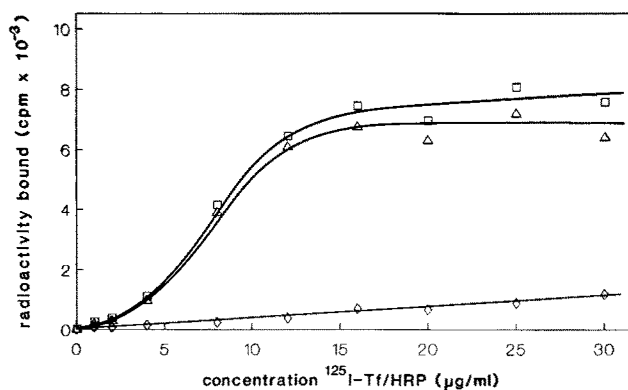

Figure 3. Saturation binding of [125I]Tf/HRP to HepG2 cells at 37°C. Tissue culture dishes (35-mm) were pre-treated as in Fig. 1, and incubated for 30 min at 37°C in 0.5 ml MEMH containing various concentrations of [125I]Tf/HRP. Excess of ligand was washed away, and the cells were dissolved in 1N NaOH. The nonspecific binding was measured in the presence of 1 mg/ml Tf. The specific binding (△) was calculated by subtracting the nonspecific binding (T) from the total binding (□).

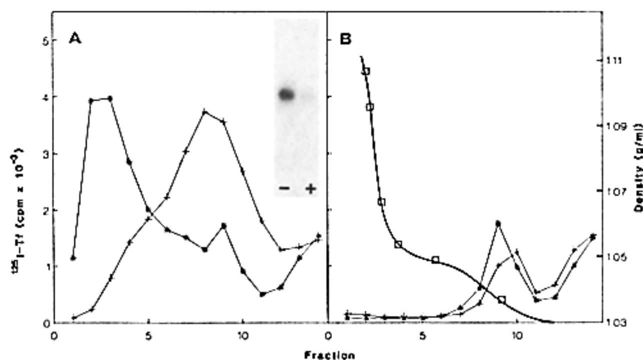

Figure 4. The effect of DAB cytochemistry on the density distribution of membrane bound ^{125}I-Tf. Tissue culture dishes (6-cm) were pre-treated as in Fig. 1. The cells were incubated for 30 min at 37°C (*A*) or 0°C (*B*) in MEMH containing both Tf/HRP and [^{125}I]Tf. Excess ligand was washed away. The cells were collected, and equal portions of the postnuclear supernatant of the homogenate were incubated with (*) or without (+) DAB and H_2O_2 before fractionation. The density of the fractions was measured with density marker beads (□). (*Inset*) Samples containing equal amounts of [^{125}I]Tf from density shifted- (fraction 2+3) (+) and non DAB treated (fraction 8+9) (−) microsomes were immunoprecipitated using rabbit anti-human Tf and were analyzed by 10% PAGE and detected by fluorography.

membrane bound radioactivity (not shown). This indicates that this peak represented plasma membrane bound ligand. Labeling at 37°C resulted in a broader peak in the Percoll gradient (mean density 1.04 g/ml) (Fig. 4 *A*). Incubation of HRP-containing microsomes with DAB and H_2O_2 before density fractionation, results in a density shift due to HRP-catalyzed polymerization of DAB inside the vesicles (Courtoy et al., 1984; Stoorvogel et al., 1987). Homogenates of cells incubated with Tf/HRP and [^{125}I]Tf were split in two equal portions, one of which was incubated in the presence of DAB and H_2O_2 before fractionation. DAB cytochemistry did not shift plasma membrane bound [^{125}I]Tf. However, endocytosed [^{125}I]Tf was shifted to fractions with a mean density of ~1.09 g/ml. The activity remaining in the fractions 8–10 could be removed by proteinase K treatment of the cells at 0°C before homogenization, indicating its plasma membrane bound origin (not shown).

In addition to the change in density of HRP-containing vesicles, DAB cytochemistry also causes cross-linking of macromolecules present in the lumen of these vesicles to the DAB reaction product, rendering them detergent insoluble (Ajioka and Kaplan, 1987). We compared the amount of immunoprecipitable [^{125}I]Tf from lysed density shifted vesicles with that of control (lysed, non-DAB treated) vesicles (Fig. 4 *A*, *inset*). The recovery of [^{125}I]Tf was reduced with ~80%. So, in addition to the effect on the density distribution, DAB cytochemistry also "cross-linked" the lumen of Tf/HRP containing vesicles.

Endocytosed Tf/HRP and Newly Synthesized AT Meet Intracellularly

The DAB density shift technique allowed us to determine the presence of newly synthesized proteins in compartments containing endocytosed Tf/HRP. We used α_1-antitrypsin (AT), a 54-kD glycoprotein, as a prototype of exocytotic proteins for two reasons: it is synthesized in a large amount by HepG2 cells, and analysis by polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE) shows a clear separation of AT molecules with high mannose oligosaccharides (hm-AT) and those with complex type oligosaccharides (c-AT) (Lodish et al., 1983). [^{35}S]Methionine pulse-chase labeling of HepG2 cells indicated that half time of maturation of hm-AT to c-AT is ~20 min, and that 50% of the glycoprotein is secreted after ~35 min of synthesis (not shown). Therefore, the c-AT molecules are on average ~15 min in the cell before secretion.

We cultured the cells for 60 min in [^{35}S]methionine containing medium to label the total secretory route of AT. Before [^{35}S]methionine labeling the cells were depleted from exogenous transferrin. During the last 10 or 30 min of [^{35}S]methionine labeling, Tf/HRP and a trace of [^{125}I]Tf were added to the medium, to continuously label the endocytotic route of the TfR. After 30 min at 37°C almost all pre-bound ligand is recycled and released into the medium (Fig. 2). Therefore continuous labeling for 30 min at 37°C was sufficient to label the whole endocytotic route. After DAB-cytochemistry, samples of the cell homogenates were fractionated on Percoll gradients. Control samples were equally treated but in the absence of DAB and H_2O_2. The density distributions of [^{125}I]Tf were similar to those in Fig. 4. From each gradient fraction [^{35}S]AT was immunoprecipitated, and analyzed by SDS–PAGE. The density distributions of [^{35}S]hm-AT and [^{35}S]c-AT containing microsomes were similar (~1.035 g/l) (Fig. 5). The total amount of [^{35}S]hm-AT compared with that of [^{35}S]c-AT recovered from the gradients is somewhat lower than expected, because little more [^{35}S]hm-AT than [^{35}S]c-AT was lost in the nuclear pellet (not shown). ^{35}S-AT present at the top of the gradient probably originated from leaky vesicles. DAB cytochemistry caused no density shift nor loss of [^{35}S]hm-AT, indicating that no significant amount of hm-AT was present in Tf/HRP containing microsomes. However, there was a considerable loss of [^{35}S]c-AT in the fractions 6 to 12. Only part of this density shifted protein was recovered in fraction 2 to 5. The rest was cross-linked to the DAB reaction product like [^{125}I]Tf (Fig. 4). The limited density shift of extractible [^{35}S]c-AT compared to that of [^{125}I]Tf might implicate that these molecules arise from vesicles containing relatively little DAB polymer, and consequently are less efficiently cross-linked. The amount of density shifted [^{35}S]c-AT was estimated by calculating the total loss of label in the fractions 6 to 12, and amounted 40 and 35% after 10- and 30-min uptake of Tf/HRP respectively. This shows that already after 10 min a maximum amount of c-AT was reached by endocytosed Tf/HRP.

The Specificity of The Procedure

To ascertain that only TfR mediated uptake of Tf/HRP caused the effect on the density distribution of [^{35}S]c-AT observed, a control experiment was performed in which an excess of non-labeled Tf was added to compete for receptor binding with both [^{125}I]Tf, and Tf/HRP (Fig. 6 *A*). Only little non-specific bound [^{125}I]Tf was found in the gradient which did not shift upon DAB treatment, indicating that all of it was plasma membrane bound and thus a negligible amount of ligand was endocytosed non-specifically. Neither loss nor any density shift of [^{35}S]c-AT was observed after

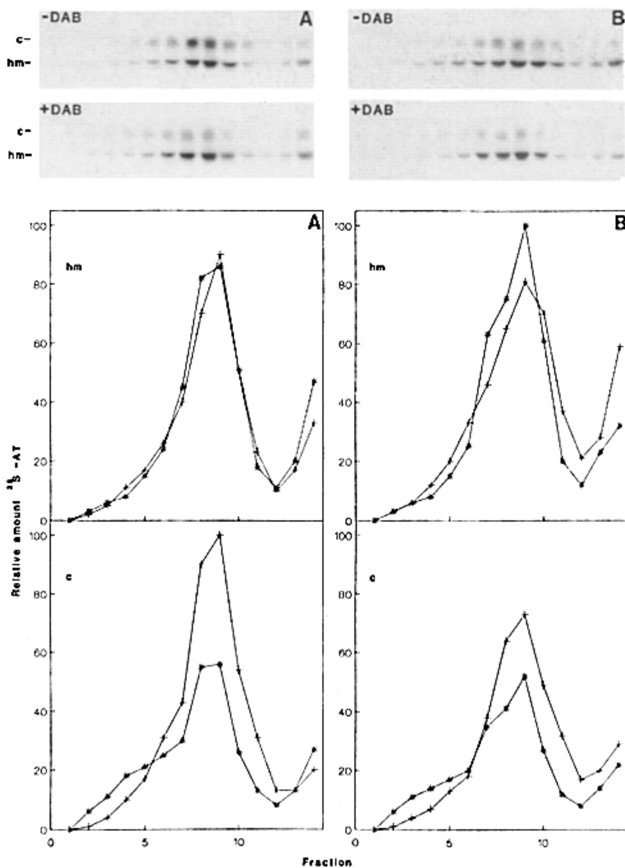

Figure 5. Tf/HPR-induced density shift and quenching of [^{35}S]AT. Tissue culture plates (9-cm) were continuously labeled with [^{35}S]methionine. During the last 10 (*A*) or 30 min (*B*) of [^{35}S]methionine labeling Tf/HRP and a trace of [^{125}I]Tf were also present in the incubation medium. The cells were washed, collected, and homogenized. Equal portions of the postnuclear supernatant of the homogenates were incubated with (*) or without (+) DAB before fractionation. From each gradient fraction [^{35}S]AT was immunoprecipitated and analyzed by SDS-PAGE (*top*). [^{35}S]hm-AT (*hm*) and [^{35}S]c-AT (*c*) Were quantitated by scanning the fluorogram, and are expressed as relative amounts. The values are only comparable within each experiment. The densities of the fractions and the distribution of [^{125}I]Tf in the gradients were similar to those in Fig. 4.

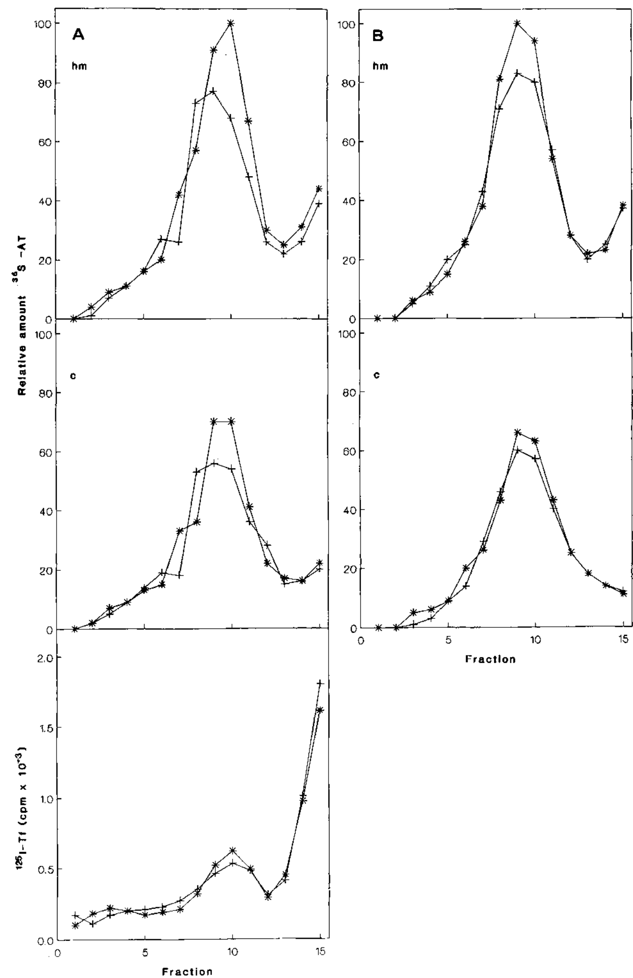

Figure 6. Specificity of the DAB-induced density shift procedure. (*A*) A 9-cm culture dish was treated as in Fig. 5 A, except that besides Tf/HRP and ^{125}I-Tf, 1 mg/ml Tf was also present in the incubation medium to prevent receptor specific Tf/HRP and [^{125}I]Tf binding. (*B*) Two culture dishes (9-cm) were treated as described in Fig. 5 *A*. One culture dish was incubated with only [^{35}S]methionine, and the other one with only Tf/HRP and [^{125}I]Tf. After washing the two cell cultures were scraped, pooled, and homogenized together. In both experiments (A+B), [^{35}S]AT was immunoprecipitated from each fraction and analyzed as in Fig. 5. [^{35}S]hm-AT (hm) and [^{35}S]c-AT (*c*) from DAB treated (*) and non-DAB treated (+) microsomes are as in Fig. 5 expressed in relative amounts. The densities of the were as in Fig. 4. The density distributions of [^{125}I]Tf in experiment B were similar to those in Fig. 4.

DAB incubation. In all experiments, the control samples of the post nuclear supernatants were incubated in the absence of both DAB and H_2O_2, because if only H_2O_2 was left out a minor density shift was observed, possibly due to endogenous H_2O_2. The incubation with DAB and H_2O_2 caused a slight increase of the total amount of [^{35}S]AT. This non-specific effect was caused by DAB monomers, and was HRP and H_2O_2 independent (not shown). If excess Tf was present in the medium, the ratio of [^{35}S]c-AT and [^{35}S]hm-AT in the fractions 6–12 remained unaltered after DAB cytochemistry, showing that in the absence of excess Tf (Fig. 5) the accessibility of [^{35}S]c-AT by Tf/HRP is TfR dependent.

The fact that [^{35}S]hm-AT never shifted in the gradient upon DAB treatment, already demonstrated the specificity of the technique. To show that DAB cytochemistry also had no effect on [^{35}S]c-AT if Tf/HRP was located in different vesicles, a mixing experiment was performed (Fig. 6 *B*). One cell culture plate was labeled for 60 min with [^{35}S]methionine, and another one for 10 min with [^{125}I]Tf and Tf/HRP. The cells were scraped, combined, homogenized, and incubated with DAB/H_2O_2. As expected, no [^{35}S]c-AT was shifted nor lost, showing that intravesicular co-localization in the cell of the secretory protein with Tf/HRP was an absolute requirement for co-shifting, and that non-specific interactions between [^{35}S]c-AT and Tf/HRP containing vesicles or non vesicular Tf/HRP cannot explain the results.

A Tf/HRP Inaccessible Pool of [^{35}S]c-AT

To further define the merging site, [^{35}S]methionine pulse-labeled cells were chased for various times and simultane-

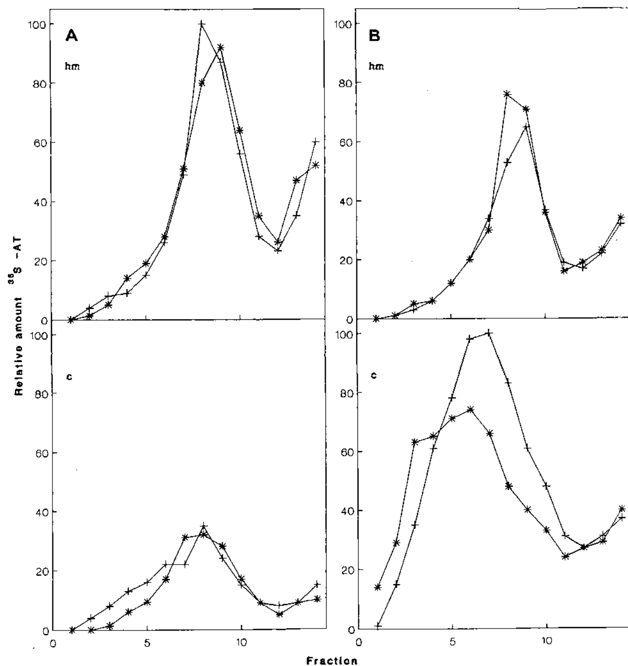

Figure 7. Tf/HRP-induced density shift and quenching of just formed [35S]c-AT and late [35S]c-AT. Tissue culture dishes (9-cm) were labeled for 10 min with [35S]methionine, and chased for 20 (*A*) or 45 min (*B*) in medium containing excess non labeled methionine. Tf/HRP and [125I]Tf were added to the culture medium at the same time as [35S]methionine (*A*) or after 15 min of chase (*B*), and in both cases were present during the last 30 min of incubation. The postnuclear supernatants of the cell homogenates were incubated with (*) or without (+) DAB and H_2O_2 before fractionation. From each fraction, immunoprecipitated [35S]AT, was analyzed as in Fig. 5. The density distributions of [125I]Tf were similar to those in Fig. 4.

ously incubated for 30 min with [125I]Tf and Tf/HRP (Fig. 7). After a chase of 20 min, only little of the total [35S]AT was complex glycosylated. This pool of [35S]c-AT was not accessible to Tf/HRP, because DAB cytochemistry had no effect on its distribution in the gradient (Fig. 7 *A*). Thus, there is a time lag between the conversion of hm-AT to c-AT and merging with Tf/HRP.

After a chase of 45 min more [35S]c-AT than [35S]hm-AT was present intracellularly (Fig. 7 *b*). The calculated amount of [35S]c-AT affected by DAB cytochemistry was ~30% (Fig. 7 *b*). This is probably an underestimation, since the vesicles containing [35S]c-AT chased for 45 min have a characteristic higher density than the vesicles containing [35S]c-AT chased for 20 min. This results in an overlap in gradient distribution of density shifted, immunoprecipitated [35S]c-AT and non-DAB incubated [35S]c-AT. The percentage of [35S]c-AT accessible to endocytosed TF/HRP could not be significantly increased in [35S]methionine pulse/chase experiments compared with experiments in which continuous labeling was used (Fig. 5). Therefore, it is likely that not all [35S]c-AT mixes with Tf/HRP before secretion.

EM Localization of Tf/HRP and Albumin

Up to here, we have obtained biochemical evidence for the simultaneous presence of endocytosed Tf/HRP and secretory protein in intracellular compartments. Next we have used electron microscopic DAB- and immunocytochemical double-labeling to localize both Tf/HRP and secretory protein containing compartments. Tf/HRP was administered during 30 min at 37°C to HepG2 cells which were preincubated in serum-free medium. The cells were labeled with DAB before cryosectioning. We immunolabeled albumin, instead of AT, because the labeling yield of the latter was too low. At the conditions used, only secretory and no exogenous albumin was labeled (Strous et al., 1983; Geuze et al., 1985; Zijderhand-Bleekemolen et al., 1987). HRP was only present as a conjugate with Tf (Fig. 1). Tf/HRP was mainly present in areas composed of tubular and vesicular organelles (Fig. 8 *A*). These structures were separate but often close to albumin-labeled Golgi areas. Therefore, we identified these structures as CURL (compartment of uncoupling receptor and ligand) (Geuze et al., 1983). We never detected DAB polymer in the Golgi stack. The loss of immunoprecipitable protein from Tf/HRP containing microsomes (Figs. 4 *A* and 5) is due to cross-linking to DAB polymer rather than to destruction of antigenic determinants (not shown). However, it is possible that some albumin present in Tf/HRP containing vacuoles was not detected, due to masking by DAB polymer. Therefore, quantitation of double-labeled compartments was not appropriate. Both HRP reaction product and albumin were only found together in a tubulo-vesicular network located in the *trans*-Golgi area (Fig. 8 *B*) (Zijderhand-Bleekemolen et al., 1987). According to its definition this compartment is termed TGR.

Discussion

This study addresses the question which compartments of the biosynthetic route are involved in the endocytosis of Tf. To this purpose we used Tf conjugated to HRP as a ligand for the TfR, and studied AT as a typical exocytotic protein. The DAB cytochemistry used to monitor co-distribution, resulted in two different signals, a density shift in a Percoll gradient of Tf/HRP containing vesicles and a reduced amount of immunoprecipitable protein from these vesicles. Both phenomena have been reported before (Courtoy et al., 1984; Stoorvogel et al., 1987; Ajioka and Kaplan, 1987). Because not all [35S]AT located in Tf/HRP containing vacuoles was cross-linked to the DAB polymer, we had to use Percoll density fractionation, and measure the loss of [35S]AT from the light fractions in the gradient to quantitate the degree of concomitance. Our results show that within 10 min of continuous endocytosis, Tf/HRP co-distributed with 40% of the total intracellular secretory c-AT. [35S]c-AT Taken up by fluid phase endocytosis is not responsible for the results for three reasons: (*a*) The percentage of medium volume, taken up by fluid phase endocytosis in 60 min is negligible (Bonsdorff et al., 1985). (*b*) If a significant quantity of [35S]AT was endocytosed, at least some would have been degraded in the lysosome. However, no loss of [35S]AT was detected and all pulse-labeled AT was eventually secreted into the medium. (*c*) If any secreted [35S]c-AT was endocytosed it would have probably been sorted immediately from recycling Tf/HRP. Trapping of intracellular located [35S]c-AT during homogenization of the cells by vesiculating Tf/HRP-binding plasma membranes cannot explain the results either. The extracellular [35S]c-AT has been washed away before homogenization.

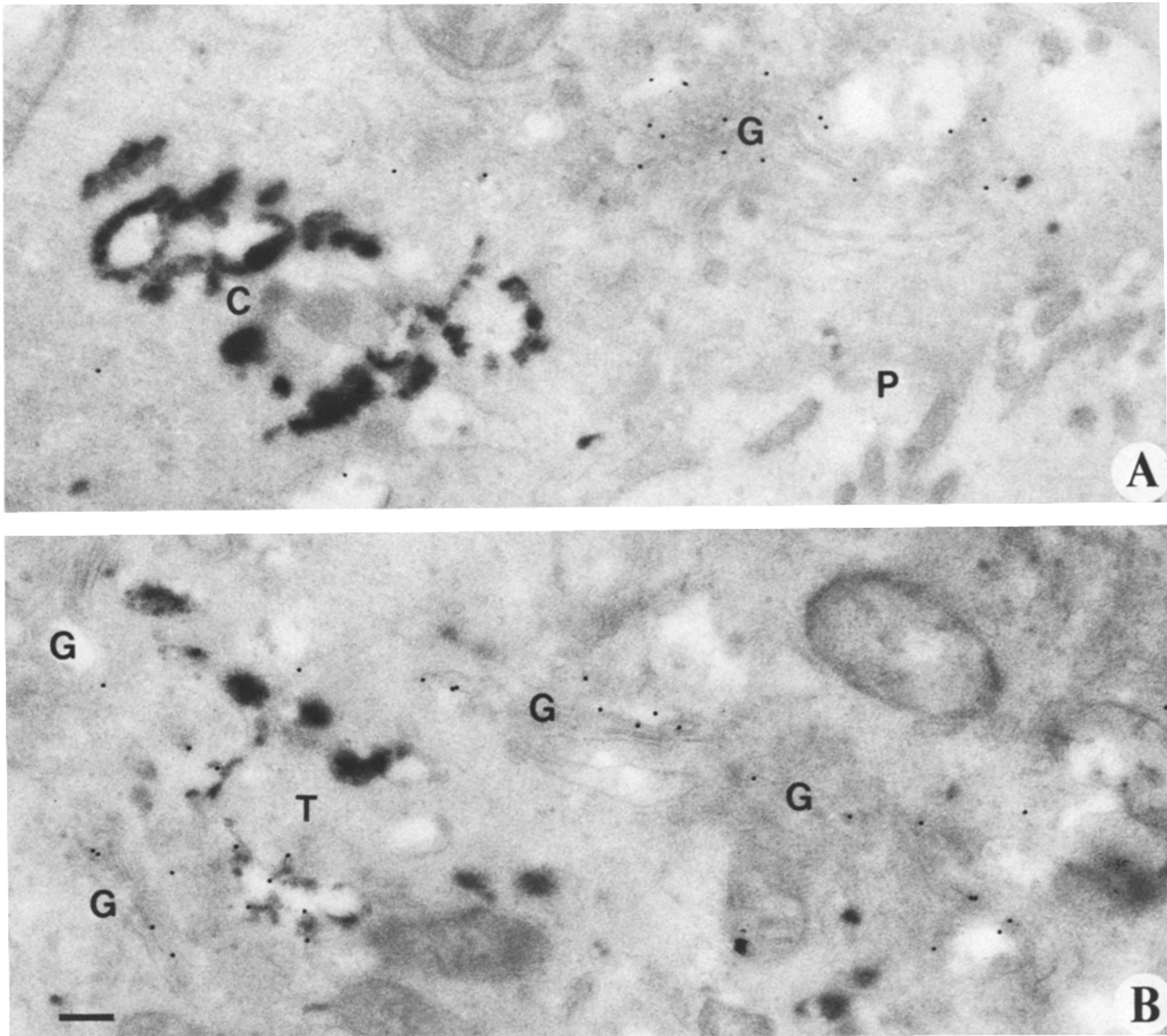

Figure 8. Ultrathin cryosections of HepG2 cells incubated for 30 min with Tf/HRP. HRP was visualized with DAB-cytochemistry. The sections were indirectly immunolabeled with 9-nm protein A-gold particles for the demonstration of endogenous albumin. The electromicrograph A shows a separate CURL (*C*) and Golgi (*G*) area labeled with DAB polymer and gold particles respectively. Plasma membrane (*P*). The electron micrograph B shows a Golgi area with stacked Golgi cisternae (*G*) enclosing profiles of the *trans*-Golgi reticulum (*T*). Albumin is present in the Golgi area and TGR, but co-localizes only in TGR with HRP reaction product. The absence of albumin labeling in the TGR like profiles with densest HRP labeling may be caused by quenching of albumin antigenicity by the DAB reaction product, Bar, 0.1 μm.

If any was plasma membrane associated, it would not shift in density after DAB incubation (Fig. 4). We also showed that neither fusion nor other non-specific interactions between microsomes containing [^{35}S]AT or Tf/HRP occurred during the procedure (Fig. 6 *B*). The accessibility of [^{35}S]c-AT by Tf/HRP was TfR dependent, because it could be completely blocked if excess Tf was also present in the culture medium.

HepG2 cells synthesize at least 20 serum proteins (Knowles et al., 1980). AT is a secretory protein which is relatively quickly secreted after synthesis. We found a half time of secretion of ~35 min (not shown). This is somewhat shorter than reported before (Strous and Lodish, 1980; Lodish et al., 1983), probably as a result of the use of different chase temperatures (37° instead of 32°C). Endo H resistance arises after the formation of N-linked oligosaccharides of the $Man_5GlcNAc_3$ out of the $Man_5GlcNAc_2$ type, which takes place in the medial Golgi cisternae (Dunphy et al., 1985) (reviewed by Kornfeld and Kornfeld, 1985). After acquisition of Endo-H resistance, exocytotic proteins in HepG2 cells need similar periods of time to be transported to the plasma membrane (Lodish et al., 1983), indicating a post-Golgi bulk-phase transport to the plasma membrane. This has been illustrated morphologically for the Vesicular Stomatitis virus membrane protein G, albumin, and newly synthesized transferrin (Strous et al., 1983). Therefore, the data

obtained in this study, showing the intravesicular mixing of secretory AT with endocytotic Tf/HRP, can probably be generalized to other exocytotic proteins produced by HepG2 cells. The morphological observation that endogenous albumin co-localizes with Tf/HRP is therefore directly comparable with the biochemical data obtained for AT. The slowly migrating forms of [^{35}S]AT in acrylamide gels are resistant to Endo H, and therefore contain N-linked Man_5-$GlcNAc_3$ or further processed oligosaccharides (Lodish et al., 1983), implicating that they must have migrated at least through the medial cisternae of the Golgi complex. Because no [^{35}S]hm-AT, nor just formed [^{35}S]c-AT was affected by DAB cytochemistry, merging of secretory proteins and Tf/HRP most likely occurred in post-Golgi compartments. In our morphological experiments we could only detect mixed populations of Tf/HRP and albumin in the TGR. No DAB reaction product was detected in the Golgi cisternae. Therefore the TGR is the most likely site of the merging of the exocytotic and endocytotic routes. The TGR is a large tubulo-vesicular network, which is possibly fractionated during cell homogenization. If Tf/HRP and [^{35}S]c-AT were both located in the TGR, but in different domains of this compartment, it is likely that they were separated into different microsomes as a consequence of the cell homogenization. This might in part account for the limited percentage of [^{35}S]c-AT affected by DAB-cytochemistry. It is clear that a significant amount of [^{35}S]c-AT remained separated from endocytosed Tf/HRP either spacially or by membranes.

Both endocytosed [^{125}I]Tf and [^{125}I]Tf/HRP recycled and were released in the medium with a half time of $\sim$10 min (Fig. 2). These kinetics are in agreement with those reported by Ciechanover et al., (1983*b*). Within this time span endocytosed Tf/HRP reached compartments of the secretory route. We could not distinguish however whether these compartments were involved in the recycling of all or just a minor part of endocytosed Tf/HRP. Several authors have suggested the existence of two different TfR recycling routes. In A431 cells Hopkins (1983) proposed a peripheral "short circuit" and a "long circuit" through the juxtanuclear area. Recently Stein et al., (1986) showed that in K-562 cells only a reduced population of TfR recycled in the presence of the proton ionophore monensin. This was explained by assuming 2 different receptor recycling routes, one of which was monensin sensitive. The authors suggested that this route could possibly merge with the biosynthetic route. Our finding that the secretory pathway in HepG2 is also perturbed with monensin (Strous et al., 1983) supports this hypothesis. Morphologically, both TfR and an endocytosed conjugate of Tf and HRP have been detected in a reticular system at the *trans*-Golgi region, but not in the Golgi stack (Willingham et al., 1984; 1985). The finding that similar structures in both KB and Vero cells could be double labeled with endocytosed ferritin conjugated Tf and exocytotic Vesicular Stomatitis viral glycoprotein (Hedman et al., 1987) indeed qualifies these structures as TGR. The Tf conjugate appeared after 30–45 min in the TGR which is much slower than overall Tf recycling, strongly suggesting that in these cell types Tf-TfR recycling through the Golgi region is a minor pathway. Contrasting to these and our results are those obtained by Woods et al., (1986) who have labeled plasma membrane TfRs of myeloma cells with specific antibodies. During endocytosis they appeared in sequence in the cis (after 5 min) and *trans*-cisternae (after $\pm$30 min) of the Golgi stack. Snider et al., (1985; 1986) showed that in K-562 cells endocytosed TfRs are modified in half times of 6 h and 2–3 h due to passage through mannosidase I and sialyltransferase containing compartments, respectively. Since the major localizations of mannosidase I and sialyltransferase are the *cis*-Golgi region (Kornfeld and Kornfeld, 1985) and TGR (Roth et al., 1985) respectively, passage in sequence through the *cis*- and *trans*-Golgi region of endocytosed TfR in K-562 cells seems unlikely. Significant transport within 30 min of Tf/HRP to the *cis*-Golgi in HepG2 cells can also be ruled out because in this time span no hm-AT was accessible for the ligand and no reaction product was detected in the Golgi stack. Possibly, cell line specificity in TfR routes can explain the conflicting data. This assumption can also be made for the asialoglycoprotein-receptor of which a significant part resides as a cycloheximide resistant pool in the Golgi stack in rat liver (Geuze et al., 1984*a*), whereas only little can be detected in the Golgi stack of HepG2 cells (Zijderhand-Bleekemolen et al., 1987).

We were not able to quantitate the amount of Tf/HRP that recycled through the TGR. The possibility of a second recycling pathway by-passing the TGR remains open. Other studies performed in other cell lines report the detection of Tf in the TGR and exocytotic coated vesicles at a slower rate than the average recycling time (Hedman et al., 1987; Fishman and Fine, 1987). These findings argue in favor of the idea that only part of endocytosed Tf is transported to compartments of the secretory route. We have shown that both asialoglycoprotein receptor-ligand uncoupling (Geuze et al., 1983) and intracellular asialoglycoprotein receptor-IgA receptor sorting (Geuze et al., 1984*b*) in rat liver occurs within an early endosomal compartment called CURL. We also showed that during endocytosis sorting of Tf and asialoglycoproteins (ligands for the asialoglycoprotein receptor, transported to the lysosome) is an intracellular event occurring immediately after uptake (Stoorvogel et al., 1987). In this study we detected endocytosed Tf/HRP in both CURL vesicles and tubules, the sorting site of recycling receptors. This could also be the site of divergence of different TfR recycling routes. There is some evidence that other plasma membrane proteins than the TfR can enter the exocytotic pathway. In both rat liver and HepG2 cells large pools of asialoglycoprotein receptor have been detected in the Golgi stack or TGR (Geuze et al., 1984*a*; Zijderhand-Bleekemolen et al., 1987). Besides receptors other membrane proteins have been detected in the biosynthetic route after being endocytosed as well (Gonatas et al., 1984; Patzak and Winkler, 1986). The bulk of plasma membrane glycoprotein in K562 cells can pass mannosidase I-containing organelles, albeit at slow rates (Snider and Rogers, 1986). Only in a few cases it has been shown that fluid phase endocytotic markers enter Golgi cisternae and the TGR (Farquhar, 1983). This study showed a connection between the exocytotic routes and endocytotic route at the level of the TGR. Whether this link is universal for other endocytotic membrane proteins remains an interesting object for further study.

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