Intracellular Interactions of Transferrin and Its Receptor during Biosynthesis

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Abstract. The interactions between transferrin (Tf) and transferrin receptor (Tfr) as they occur during biosynthesis were studied in the human hepatoma cell line HepG2, which synthesizes both. Early during biosynthesis the Tfr monomer is converted to a disulfide-linked Tfr dimer. The Tfr monomer is not able to bind Tf, but Tf binding is observed as soon as the covalent Tfr dimer is formed and can take place in the ER. The Tf-Tfr complex is transported through the Golgi reticulum and *trans*-Golgi reticulum (TGR) and is ultimately delivered to an acidic compartment, where Tf releases its Fe^{3+}. We did not observe conversion of Tf to apoTf in the TGR, showing that the part of the TGR passed by secreted Tf has a pH higher than 5.5.

We conclude that when a ligand-receptor combination is synthesized by one and the same cell, ligand and receptor can interact during biosynthesis and be transported to the cell surface.

INTERACTIONS of ligands with receptors are essential in the communication of cells with their surroundings, and furthermore supply the cell with nutrients. When a cell produces both a soluble polypeptide ligand and its receptor, they usually follow the same biosynthetic route. Examples of receptors and corresponding ligands synthesized by the same cell include the couples IL-2/IL-2 receptor, transferrin/transferrin receptor (Tf/Tfr),[1] apolipoprotein E/LDL (ApoE/LDL) receptor, and transforming growth factor α/epidermal growth factor receptor (TGFα/EGFR) (for reviews see references 5, 7, 17, 22, 33). In principle, the simultaneous production of receptor and ligand can result in their binding in the course of biosynthesis. Its consequences may be several, depending on the type of ligand and receptor. When the ligand is a growth factor, binding of the ligand to its receptor during biosynthesis can result in arrival of the ligand–receptor complex at the cell surface, and may produce a growth stimulus for the cell. This situation constitutes a special case of an autocrine loop: secretion of the otherwise released growth factor is not necessary. Autocrine loops have been postulated to operate for TGF, EGF, and PDGF with their respective receptors in different cell lines, and figure prominently amongst the models explaining tumor growth (8).

When the ligand supplies a cell with certain nutrients (e.g., Tf, LDL), binding of ligand and receptor during bio-synthesis can likewise result in appearance of the complex at the cell surface followed by its internalization. The ligand will thus be delivered back to the cell from which it originated.

To our knowledge, the interaction of receptors with their soluble polypeptide ligands in the course of biosynthesis has not been documented. We studied the interactions of Tf with Tfr during biosynthesis in HepG2 cells, because Tf-Tfr interactions at the cell surface and during recycling have been particularly well defined (1, 7, 14, 17, 35). When Tf binds to Tfr at the cell surface, the Tf-Tfr complex is internalized and arrives in an acidic endosomal compartment. There Tf releases its bound Fe^{3+}, but the resulting apotransferrin (apoTf)-Tfr complex is stable at the resident endosomal pH (pH <5.5). The ApoTf-Tfr complex recycles back to the cell surface where it dissociates, because at neutral pH the apoTf-Tfr complex is not stable. apoTf is released, and Tfr can then start a new cycle (7, 17).

Ligand–receptor interactions during biosynthesis can be studied if such complexes can be isolated. For Tf-Tfr interactions this can be achieved by coimmunoprecipitation of Tf with Tfr, using an anti-Tfr antibody. We show that when the Tf-Tfr ligand-receptor combination is synthesized by one and the same cell (HepG2), Tf can interact with Tfr during biosynthesis and the Tf-Tfr complex is ultimately delivered to a compartment of acidic pH where Tf releases its Fe^{3+}. The resulting apoTf-Tfr complex will dissociate at neutral pH. The *trans*-Golgi reticulum (TGR) does not constitute the compartment where Tf is converted to apoTf.

A ligand–receptor combination that reaches the cell surface through the biosynthetic route will behave similarly to a receptor occupied by ligand added externally.

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1. *Abbreviations used in this paper:* α_1AT, alpha$_1$-antitrypsin; apoE, apolipoprotein E; apoTf, apotransferrin; ASGR, asialoglycoprotein receptor; dNM, 1-deoxynojirimycin; EGFR, epidermal growth factor receptor; 1D-IEF, one-dimensional isoelectric focussing; NANAse, neuraminidase; PDGF, platelet-derived growth factor; Tf, transferrin; Tfr, transferrin receptor; TGFα, transforming growth factor α; TGR, *trans*-Golgi reticulum.
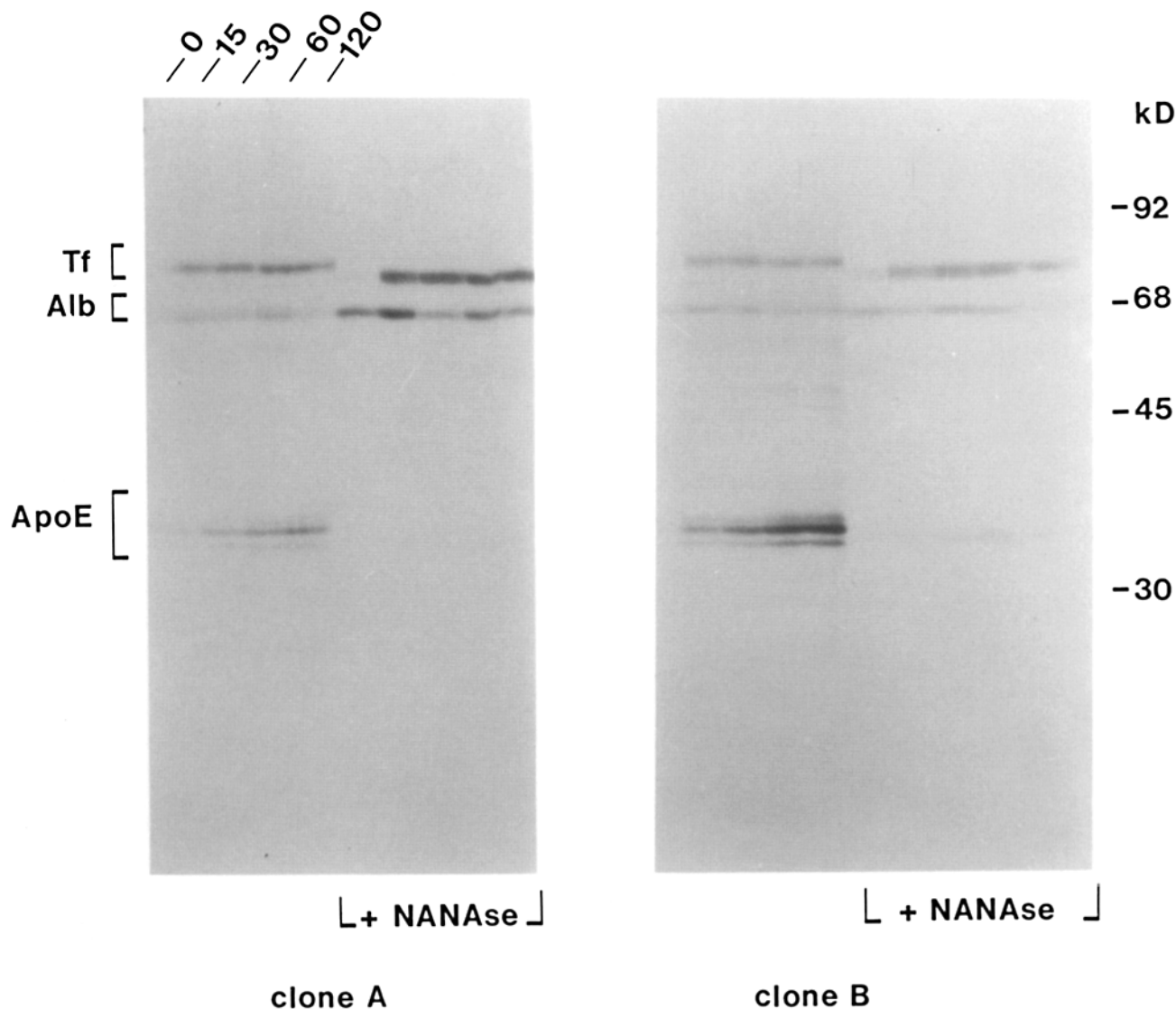
Figure 1. Uptake of proteins secreted by HepG2. HepG2 cells (two different clones) were allowed to take up secreted proteins, isolated from ^{35}S-labeled HepG2 cells, for different times indicated in minutes above the gel. Total cell lysates, including bound, radiolabeled proteins, were analyzed on SDS-PAGE. Three major proteins bind to HepG2 cells: apolipoprotein E (*ApoE*) via the apoB/E receptor, albumin (*Alb*), and transferrin (*Tf*) via the transferrin receptor. The identity of the proteins indicated, was established by immunoprecipitation. NANAse digestion of the radiolabeled secreted proteins produced by HepG2 virtually abolishes apoE uptake but not Tf uptake. Note the steady-state levels of bound Tf from 15 min on, consistent with rapid recycling of Tfr. The position of the markers are indicated.

Materials and Methods

Cells and Cell Culture

The human hepatoma cell line HepG2 (18) was grown in DME supplemented with 10% (vol/vol) FCS. The human erythroleukemia cell line K562 (20) was grown in RPMI-1640 supplemented with 10% (vol/vol) FCS.

Antibodies

The following antibodies were used. Rabbit anti–human transferrin serum and rabbit anti–human α_1 antitrypsin serum (Central Laboratories of the Red Cross Bloodtransfusion Centre, Amsterdam, The Netherlands), mouse anti–human transferrin receptor monoclonal antibody (66Ig10) (36).

Gel Electrophoresis

SDS gel electrophoresis (9) and one-dimensional isoelectric focussing (1D-IEF) (24) were performed as described. Gels were fluorographed using DMSO/PPO and exposed on Kodak XAR5 films.

Preparation of [^{35}S]Methionine-labeled HepG2 Supernatant

HepG2 cells were cultured on Ham F-12 medium 20 h before labeling, followed by labeling with [^{35}S]methionine in methionine-free RPMI medium for 6 h. The supernatant was removed and concentrated through a Centricon 30 (Amicon Corp., Danvers, MA), followed by removal of free [^{35}S]methionine by gel filtration on a PD-10 column in water. The resulting preparations were stored at 4°C.

Neuraminidase treatment of HepG2 supernatants was performed at room temperature using 25 U of neuraminidase (type V; Sigma Chemical Co., St. Louis, MO) per ml.

Uptake of [^{35}S]Methionine-labeled Proteins

HepG2 cells were plated in 5-cm petri dishes in DME plus 10% FCS. HepG2 cells were then exposed for 24 h to serum-free DME supplemented with 20 mM Hepes, 10 μM 17-β-estradiol, 30 nM Na-selenite, 40 ng/ml dexamethasone, and 10 μg/ml insulin. The cells were washed three times with Hank's medium and exposed to 1 ml of the above described preparation

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of radiolabeled HepG2 secretory proteins. Samples (individual dishes) were taken at 0, 15, 30, 60, and 120 min. Medium was removed and the cells were washed three times at 4°C with PBS (Dulbecco). Cells were then lysed in 1 ml NP-40 lysis mix (10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.5% (vol/vol) NP-40, and 1 mM PMSF). Debris was removed (5 min, 10,000 g) and the supernatant was lyophilized. The residue was resuspended in 30 μl H_2O and 15 μl 2× SDS sample buffer was added. Samples were boiled and analyzed by SDS-PAGE on a 10% gel.

Immunoprecipitation of Ligand and Receptor

HepG2 cells were exposed to radiolabeled HepG2 supernatant (containing Tf) for 1 h at 0°C in PBS or DME (which contains Fe^{3+} that should allow loading of apoTf and its conversion to Tf). Nonbound material was removed by washing with PBS (three times) at 0°C.

Cells were then lysed at either pH 5.1 (50 mM NaAc pH 5.1, 5 mM $MgCl_2$ and 0.5% [vol/vol] NP-40) or pH 7.4 (50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, and 0.5% [vol/vol] NP-40). Tfr and Tfr-Tf were immunoprecipitated with the mAb 66Ig10 (36) from lysates prepared at pH 5.1 or 7.4. Residual Tf was immunoprecipitated with the rabbit anti-human transferrin serum.

Immunoprecipitates from cells lysed at pH 5.1 were washed in 50 mM NaAc pH 5.1, 5 mM EDTA, 150 mM NaCl, and 0.5% (vol/vol) NP-40. Immunoprecipitates from cells lysed at pH 7.4 were washed in a solution of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA and 0.5% (vol/vol) NP-40 (NET buffer). Immune complexes were resuspended in SDS sample mix, boiled, and analyzed by SDS-PAGE on a 10% gel.

Pulse–Chase Experiments

Pulse–chase experiments were performed as described (25). Binding of secreted radiolabeled Tf to HepG2 cells during the chase was prevented by inclusion of nonradioactive Tf in the labeling medium to a final concentration of 10 μg/ml. For each chase point, one 5-cm petri dish of semiconfluent HepG2 cells was used. Cells were pulsed with [^{35}S]methionine for the times indicated in the results, and incorporation of label was terminated by addition of nonradioactive methionine to a final concentration of 1 mM. Where indicated, chloroquine (100 μM) was added at the beginning of the chase. Cells were lysed in lysis mix (see above) containing 10 μg of nonradioactive Tf per ml. The immunoprecipitations were performed following a strict timetable, resulting in the final washed immunoprecipitate within 4–5 h after lysis of the cells. However, prolonged incubation times during immunoprecipitation (up to 24 h) did not affect the amount of radiolabeled Tf coprecipitated with Tfr (data not shown). Tf from Tf-Tfr complexes was isolated by boiling the Staphylococcus aureus pellet in 20 μl 2% (wt/vol) SDS for 5 min. NET buffer (950 μl) was added, and the supernatant was precleared twice with normal rabbit serum and Staphylococcus aureus. Tf was then immunoprecipitated using the rabbit anti-human transferrin serum.

Endo H digestions were performed as described (25). NANAse digestion on immunoprecipitates were performed as described (24).

Tfr Binding to Immobilized Tf

K562 cells were labeled with [^{35}S]methionine for 60 min, and used as a source of Tfr. Cells were lysed using NP-40 lysis mix (pH 7.4). Debris was removed and the lysate was precleared with Sepharose. The lysate was incubated with Tf-Sepharose (prepared by coupling Tf to CNBr-activated Sepharose) at 0°C (overnight) in the presence or absence of 10 μg/ml competing Tf. After removal of Tf-Sepharose, Tfr was immunoprecipitated from the unbound fraction with the mAb 66Ig10 (36).

The Tf-Sepharose-bound material was washed three times with NET-buffer (pH 7.4) followed by three washes and a 1-h incubation (4°C) with buffer containing 50 mM NaAc (pH 5.1), 5 mM EDTA, and 150 mM NaCl. The eluted material was collected and Tfr was immunoprecipitated from it using 66Ig10 (36). The Tf-Sepharose was subsequently washed with NET buffer (pH 7.4) and again Tfr was immunoprecipitated from the eluate. The immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions.

Conversion of apoTf during Secretion

Two dishes of semiconfluent HepG2 cells were labeled with 125 μCi [^{35}S]methionine in methionine-free RPMI-1640 medium for 4 h in the absence or presence of chloroquine (100 μM). To assess the possible role of free Fe^{3+} in the medium, the identical experiment was performed including 10 μM $NaVO_3$ in the medium. K562 cells (4 × 10^7) were washed twice with PBS (0°C). Radiolabeled secretory proteins (containing Tf) obtained from HepG2 cells were added to the K562 cells and incubated for 1 h at 0°C. The K562 cells having bound Tf were washed twice with PBS (0°C), lysed in NP-40 lysis mix (pH 7.4) and Tfr was immunoprecipitated using the mAb 66Ig10 (36). The unbound Tf was immunoprecipitated from the supernatant with the rabbit anti-human Tf serum.

Results

Secretion and Uptake of Glycoproteins by HepG2

HepG2 cells synthesize and secrete considerable quantities of Tf (18). As in every rapidly dividing cell line, Tfr is also abundantly expressed. In addition, HepG2 cells are positive for the asialoglycoprotein receptor (ASGR) (4, 10). Because many proteins are synthesized with N-linked glycans, these proteins should be a ligand for the ASGR during passage
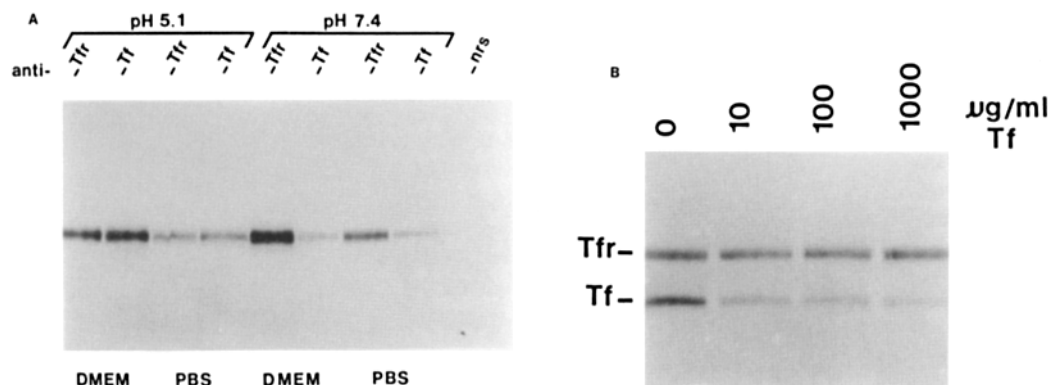

Figure 2. Tf and Tfr can be coimmunoprecipitated. (A) Coimmunoprecipitation of ligand and receptor. Radiolabeled HepG2 supernatant was added to HepG2 cells at 0°C in DME or PBS. Nonbound proteins were removed, the cells were washed and were subsequently lysed at pH 5.1 or 7.4. Tfr was immunoprecipitated with the MAb 66Ig10 followed by a sequential immunoprecipitation of free Tf with anti-Tf serum. The immunoprecipitates were analyzed by SDS-PAGE on a 10% gel. Note the difference in efficiency of coimmunoprecipitation of Tf with Tfr at pH 5.1 and 7.4 nrs; normal rabbit serum control. (B) Binding of Tf to Tfr during lysis. HepG2 cells were labeled with [^{35}S]methionine for 15 min and chased for 60 min. To exclude that Tf binds to Tfr after lysis of the cells, HepG2 cells were lysed in lysis mix containing different concentrations of nonradioactive Tf as indicated above the figure. Tf-Tfr was immunoprecipitated with the monoclonal antibody 66Ig10 and analyzed by SDS-PAGE on a 10% gel.

through the *trans*-Golgi apparatus, provided they have a terminal galactose residue (10). Finally, apoE and the LDL receptor, both synthesized by HepG2 cells (34), constitute a similar couple of receptor and corresponding ligand (13).

To establish which of these ligand–receptor interactions can be visualized using metabolically labeled ligands, radiolabeled proteins secreted by HepG2 cells were offered to an unlabeled culture of HepG2 cells. At different time points, samples of these cultures were taken, and analyzed by SDS-PAGE for the proteins retained from the mixture of radioactive proteins offered. Surprisingly, the pattern of polypeptides detected was remarkably simple (Fig. 1), and could be explained by the presence of Tf, albumin, and apoE, as determined by immunoprecipitation with the relevant antisera (data not shown). Albumin sticks nonspecifically to membranes (28). Indeed, the extent of albumin binding did not show any systematic variation with time or temperature (data not shown). The kinetics of uptake of Tf and apoE were widely different: a continuous increase in the amount of apoE was observed until 120 min, whereas the uptake of Tf had reached a plateau already after 15 min. These observations are consistent with internalization of apoE via the LDL-receptor (13), and Tf entering the Tf cycle (6, 16, 17). Because of the rapid cycling time of Tfr (8–15 min) (6), a steady-state distribution of radiolabeled Tf over the intra- and extracellular compartments is rapidly attained.

Prior exposure of radiolabeled proteins secreted by HepG2 to neuraminidase (NANAse) abolished almost completely uptake of apoE, but not binding of albumin or uptake of Tf. Contrary to expectation, no increase in uptake or binding of major desialylated glycoproteins to ASGR was observed. Whether the two clones of HepG2 cells (obtained from different investigators) both express only low levels of ASGR, or whether the native, NANAse-treated, HepG2 secretory proteins are inferior substrates for the ASGR remains to be established. Efficacy of NANAse treatment was checked by 1D-IEF analysis of Tf and α_1-antitrypsin (α_1-AT) (data not shown), and can also be assessed from the difference in the relative molecular mass for NANAse-exposed and control Tf (Fig. 1). For the remainder, we focussed our attention on the Tf–Tfr couple, because of the high levels expressed by HepG2 and of the availability of reagents recognizing Tf and Tfr.

Removal of sialic acids in no way interfered with uptake of Tf (29). Moreover, Tf produced by cells in the presence of the mannosidase inhibitors 1-deoxynojirimycin (dMM) or swainsonine (11) was likewise taken up efficiently (data not shown). We conclude that the type of N-linked carbohydrates carried by Tf is not a factor that determines its ability to bind to Tfr. This point is of relevance to our biosynthetic experiments described below.

Immunoprecipitation of the Tf–Tfr Complex

We established the conditions for the recovery of Tf–Tfr complexes by immunoprecipitation at two different pH values. At neutral pH, only the Tf–Tfr complex is stable, whereas at acidic pH (pH <5) the Tf in the Tf–Tfr complex is converted to apoTf. The apoTf–Tfr complex is stable at acidic pH (7, 17). In the course of the normal Tf cycle, the Tf–Tfr complex reaches the acidic compartment where Fe^{3+} is released from Tf, resulting in the conversion of Tf to apoTf. As long as the receptor–ligand complex is in a com-

partment of low pH it is stable: upon return to the cell surface, and thus exposure to neutral pH, apoTf dissociates from Tfr (7, 17).

HepG2 cells were exposed to ^{35}S-labeled Tf-containing protein mixture (labeled HepG2 supernatant) in PBS (containing no Fe^{3+}) or DME (containing Fe^{3+}). Lysates were prepared at pH 5.1 (where Tf–Tfr is converted to apoTf–Tfr, which is stable at pH 5.1) and at pH 7.4, where only Tf–Tfr is stable. The anti–Tfr antibody 66Ig10 (36) recovers Tfr-bound Tf under all experimental conditions tested. Cells maintained in DME invariably give higher recovery of bound Tf, presumably because compared to cells maintained in PBS a smaller proportion of the available Tf is in the apo form, which is unable to bind to Tfr at neutral pH (Fig. 2 *A*).

A second round of immunoprecipitation with a polyclonal rabbit anti–human Tf antiserum yields free Tf not in a complex with Tfr. This fraction is greater when lysis is carried out at pH 5.1. The small amount of Tf isolated in the second round of immunoprecipitation at neutral pH could represent Tf bound to molecules other than Tfr or molecules derived from Tf–Tfr complexes disrupted in the course of lysis. Thus, immunoprecipitation at pH 5.1 and at pH 7.4 allows the isolation of ligand–receptor complexes, but the stability of this complex is lower at pH 5.1.

Because lysates of biosynthetically labeled HepG2 contain both Tfr and free Tf, precautions must be taken to prevent association of free radiolabeled Tf with Tfr. For example, this situation could arise when radiolabeled Tf present in the ER could combine after lysis with Tfr present in a different subcellular compartment. To establish whether this possibility indeed applies, HepG2 cells were pulse labeled for 15 min and chased for 60 min. Immunoprecipitation of Tfr was then carried out in the presence of increasing amounts of competing nonradioactive Tf. The amount of Tf complexed with Tfr was reduced by the inclusion of 10 μg/ml of nonradiolabeled Tf, suggesting that some association of receptor and ligand did indeed take place in the lysate (Fig. 2 *B*). Raising the concentration of competitor Tf to 100 μg/ml did not further reduce the amount of radiolabeled Tf coimmunoprecipitated. We therefore routinely included 10 μg/ml of competing Tf in our lysis mix in subsequent experiments.

Tf–Tfr Interaction During Biosynthesis

To investigate the interaction of Tf and Tfr in the course of biosynthesis, a series of pulse–chase experiments was carried out (Fig. 3). Analysis of the immunoprecipitates prepared at pH 7.4 with the anti–Tfr mAb 66Ig10 show rapid transport of Tfr to the *trans*-Golgi apparatus within 30 min after synthesis, when Tfr becomes resistant to Endo H digestion (Fig. 3 *A*). Mature Tfr contains two complex and one high-mannose carbohydrate chain and is therefore always partially susceptible to Endo H treatment (27, 31). After a 15-min chase, labeled Tf is coimmunoprecipitated with Tfr. The identity of Tf was proven by dissociation of the anti–Tfr immunoprecipitates, followed by a reprecipitation with the rabbit anti–human Tf serum (Fig. 3 *B*). In the Tfr-bound Tf fraction, no modification to complex-type N-linked glycans is seen. Analysis of the remaining free Tf present in the lysates shows the appearance in time of Tf of a higher apparent molecular weight, comigrating with Tf recovered from the supernate (Fig. 3 *B*). This Tf contains complex-type N-linked glycans only. Note that the amount of Tfr recovered
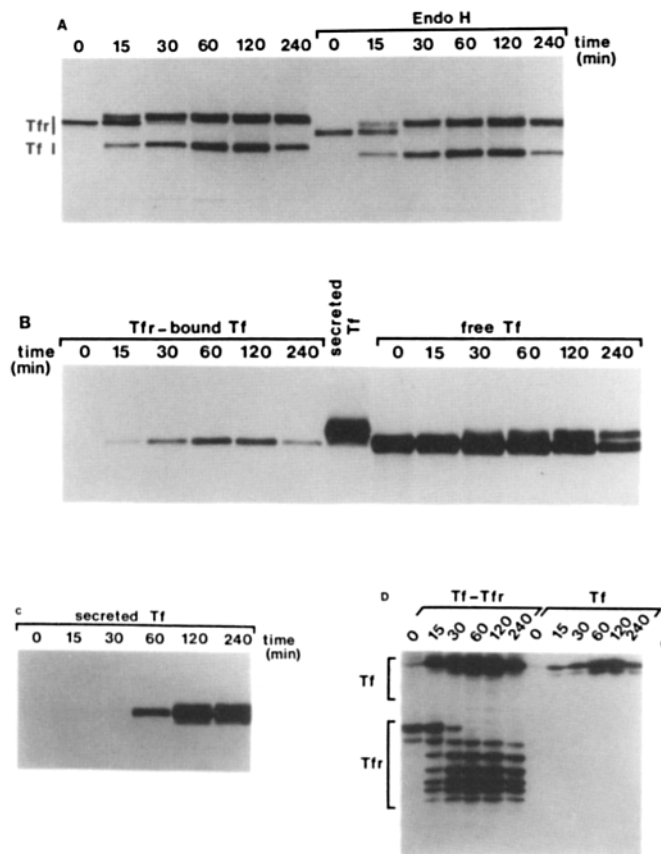
Figure 3. Binding of Tf to Tfr during biosynthesis. (*A*) Pulse–chase analysis of the Tf association with Tfr. HepG2 cells were labeled with [^{35}S]methionine for 15 min and chased for the times indicated above gel. (*Left*) Tfr is immunoprecipitated from the lysates and analyzed by SDS-PAGE on a 10% gel. Tfr shift to a higher apparent molecular weight at 15 min of chase. From 15 min of chase Tf can be immunoprecipitated with Tfr. The amount of Tf immunoprecipitated with Tfr increases up to 60 min and decreases after 120 min of chase. The position of Tf and Tfr are indicated. The identity of Tf was proven by dissociation of the Tf–Tfr complex followed by isolation of Tf by immunoprecipitation (see *B*). (*Right*) The immunoprecipitates shown in the left panel were digested with Endo H. The Tf isolated with the Tfr complex is fully Endo H sensitive and therefore intracellular. Mature Tfr contains one high-mannose and two complex-type carbohydrate chains and therefore remains partially sensitive for Endo H. (*B, left*) Tfr-bound Tf. The Tfr immunoprecipitates as shown in *A* (*left*) were denatured, and Tf was immunoprecipitated with a rabbit anti–human Tf serum. The migration of Tfr-bound Tf is compared with that of secreted Tf. Tfr-bound Tf migrates faster than secreted Tf. (*Right*) Analysis of free intracellular Tf. Remaining free Tf was immunoprecipitated from the lysates after the first round of immunoprecipitation of Tfr. Most newly synthesized Tf do not bind to Tfr (compare with *A*). Note the conversion of Tf to a higher apparent molecular weight after 30 min of chase that is not visible with Tf recovered in the Tfr–Tf complex. (*C*) Secretion of Tf. Tf was immunoprecipitated from the supernatants of pulse-labeled HepG2 cells. Secretion of Tf starts at ~60 min of chase. (*D*) Analysis of Tf and Tfr on 1D-IEF. The immunoprecipitated Tf–Tfr complex (see *A, left*) and Tf isolated from the immunoprecipitated Tf–Tfr complex (see also *B, left*) were analyzed on 1D-IEF. The position of Tf and Tfr are indicated. Sialylation of Tfr (indicating arrival in the *trans*-Golgi apparatus) results in a shift to a more acidic position on 1D-IEF and is visible after 15 min of chase. Hardly any sialylation of Tf bound to Tfr is observed (see also Fig. 4 *A*). Cathode is at the top.

by the mAb 66Ig10 increases towards the later time points of chase. The 66Ig10 antibody was raised against intact cells, and thus presumably dimeric receptor. It is possible that the affinity of 66Ig10 is higher towards the dimeric forms of Tfr.

The immunoprecipitates shown in Fig. 3, *A* and *B* were also analyzed by 1D-IEF to allow the detection of charge modifications of Tf and Tfr. Already after 15 min of chase, Tfr arrives in the *trans*-Golgi apparatus as monitored by a shift on 1D-IEF, due to the addition of (negatively charged) sialic acids (Fig. 3 *D*) (3, 30). This modification is essentially complete after 60 min of chase. No such modification is seen for Tf, in agreement with the results of Fig. 3, *A* and *B*.

The amount of Tf recovered in the anti-Tfr immunoprecipitates prepared at pH 7.4 increases from 15 until 120 min of chase and subsequently decreases (Fig. 3 *A*). This decrease represents loss of Tf from the Tf–Tfr complex due to events described below. Tf bound to Tfr is fully Endo H sensitive (Fig. 3 *A*), showing that binding of Tf to Tfr does take place intracellularly. Secreted Tf is Endo H resistant (data not shown) and fully sialylated (Fig. 4 *A*). The early binding of Tf (at 15 min of chase) at which no secretion of Tf can be detected (Fig. 3 *C*), is further proof that this interaction takes place intracellularly.

The data shown thus far establish that Tf can interact with Tfr during biosynthesis, and that the bound Tf is released subsequently from the Tf–Tfr complex. Where does this dissociation take place? Tf is converted to apoTf in subcellular compartments with a pH lower than 5.5 (7, 17). In principle,

this could occur in (parts of) the TGR; its resident pH is not easily established, but is known to be slightly acidic (2, 14). If Tf were converted to apoTf in the TGR, then the Tf–Tfr complex likewise will be converted there to apoTf–Tfr, and dissociate upon arrival at the cell surface. Otherwise, it is the Tf–Tfr complex that will arrive at the cell surface, which may then enter the usual Tf cycle. Another explanation is that there is a direct transfer of the Tf–Tfr complex produced biosynthetically to an intracellular compartment that is part of the endocytic pathway. This would also result in delivery of Tf–Tfr complex to an acidic compartment.

To show that an acidic compartment is involved in the dissociation of the biosynthetically labeled Tf–Tfr complex, a pulse–chase experiment was performed in the absence or presence of 100 μM chloroquine, a compound that neutralizes acidic compartments. Cells were lysed in a buffer of pH 7.4 or 5.1. Immunoprecipitation at pH 7.4 results in the recovery of the Tf–Tfr complex only, whereas immunoprecipitation at pH 5.1 results in the conversion of Tf–Tfr and the recovery of all complexes in the apoTf–Tfr form. To improve the signal-to-noise ratio, the isolated Tf–Tfr complex was dissociated by SDS treatment, and Tf was immunoprecipitated at pH 7.4. The immunoprecipitates were analyzed by 1D-IEF to visualize the sialylated forms of Tf: they represent mature Tf. (Tf contains two complex N-linked glycans [37] with one to four sialic acids; an average of two to three sialic acids per glycan [Fig. 4 *B*].) Neutralization of acidic compartments with chloroquine results in a considerably
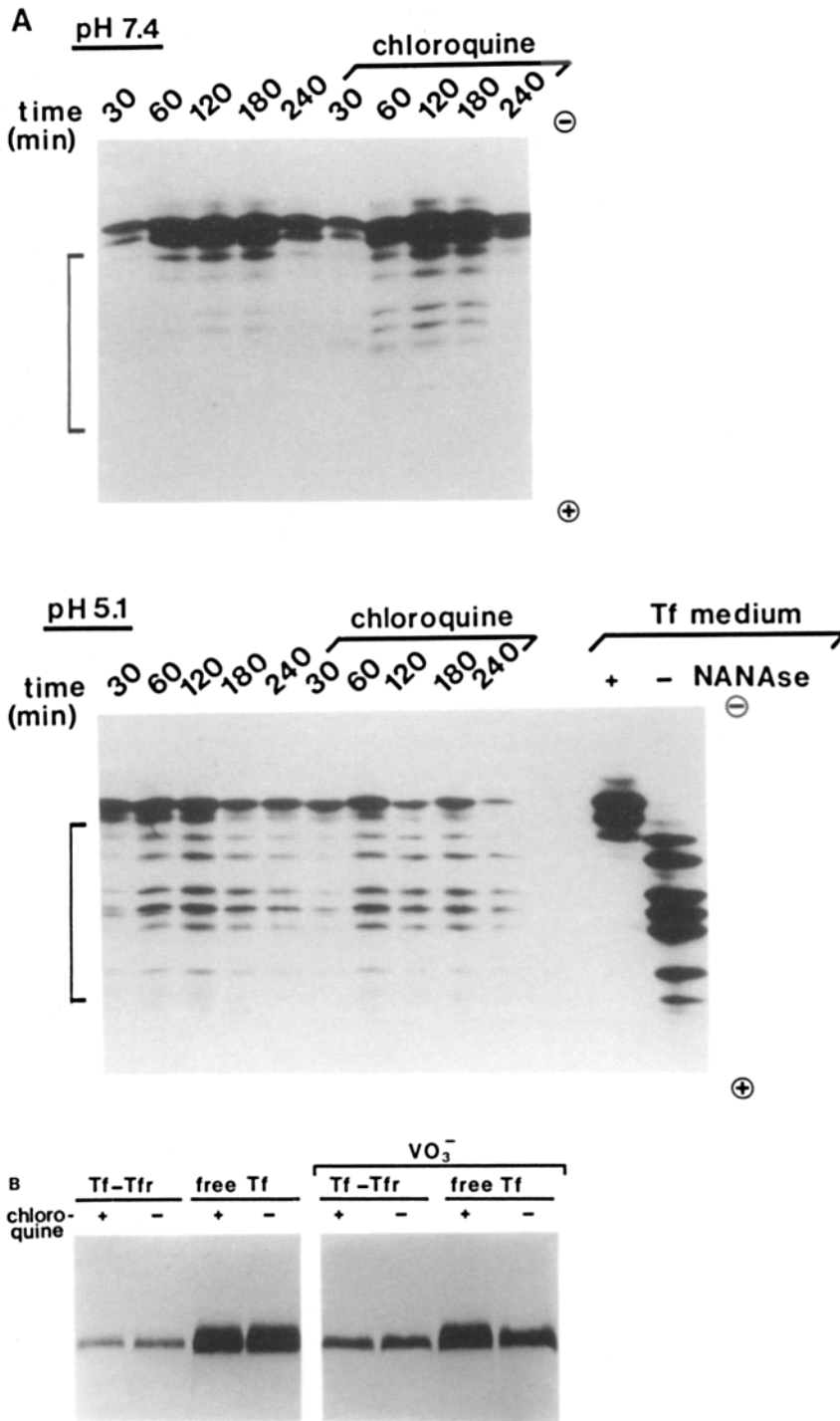
A pH 7.4

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Figure 4. Dissociation of Tf-Tfr complex after passage of an acidic compartment. (*A*) Stability of Tf-Tfr and apoTf-Tfr through passage of an acidic compartment. HepG2 cells were labeled with [^{35}S]methionine for 15 min and chased for the times indicated. Where indicated chloroquine was added at the beginning of the chase. The Tfr-Tf complex was immunoprecipitated with the anti-Tfr mAb 66Igl0 from equal amounts of TCA radioactivity in lysates at pH 7.4 (*top*) or 5.1 (*bottom*). The immune complexes were dissociated by exposure to SDS at 95°C and Tf derived from the Tfr-Tf complex was immunoprecipitated and analyzed by 1D-IEF. To show the position of sialylated and non-sialylated Tf, labeled Tf isolated from the medium was analyzed in parallel before or after digestion with NANAse. The position of sialylated Tf is indicated by brackets. The presence of sialylated Tf indicates passage of Tf through the *trans*-Golgi complex. Clearly, more sialylated Tf-Tfr is recovered when acidic compartments are neutralized. However, the amounts of Tf-Tfr including apo-Tf-Tfr recovered (at pH 5.1) are comparable upon chloroquine treatment. Cathode is at the top. (*B*) Tf is not converted to apoTf in the TGR. HepG2 cells were labeled with [^{35}S]methionine for 3 h in the presence or absence of 100 μM chloroquine. A separate experiment was performed including NaVO₃ in the medium because VO₃⁻ precipitates Fe^{3+} cations. K562 cells were then incubated with the radiolabeled HepG2 supernatant at 0°C for 1 h, the nonbound fraction was removed, and the Tfr-Tf complex was immunoprecipitated from K562 lysates. Tf was immunoprecipitated from the nonbound fraction. Immunoprecipitates were analyzed by SDS-PAGE on 10% gel.

higher recovery of sialylated Tf bound to Tfr when isolated at pH 7.4 (Fig. 4 *A, top*). Thus an acidic compartment is involved in the dissociation of Tf from Tfr.

To establish whether the release of Tf from Tfr is preceded by conversion of Tf-Tfr to apoTf-Tfr, we isolated Tfr at pH 5.1. At pH 5.1, the apoTf-Tfr complex is recovered. In contrast to lysis at pH 7.4, lysis at pH 5.1 showed a considerable recovery of sialylated (apo)Tf bound to Tfr (Fig. 4 *A, bottom*) in comparable quantities for control- and chloroquine-treated cells. The Tf complexed to Tfr and isolated at pH 5.1 but not at pH 7.4 must therefore be apoTf. Tf bound to Tfr

in cells exposed to chloroquine will ultimately be released from Tfr by competition with Tf present in the medium (17).

That conversion of Tf to apoTf does not take place in the secretory pathway (TGR) is based on the following experiment. Binding of Tf to Tfr on the cell surface of intact erythroleukemic K562 cells was used to assess the relative amounts of secreted Tf and apoTf from control and chloroquine-treated cells. Inclusion of choloroquine in the HepG2-labeling medium did not produce a shift in the amount of Tf bound to K562 cells relative to the unbound fraction, which must include apoTf. Inclusion of VO₃⁻ in the labeling

medium to complex any free Fe^{3+} produced an equivalent result: under the latter conditions, conversion of apoTf to Tf in the medium is precluded (Fig. 4 *B*). Had conversion of Tf to apoTf occurred in the secretory pathway in untreated HepG2 cells, no Tfr-bound material would have been expected. However, binding of Tf to the K562 Tfr is observed, and thus we conclude that the TGR is not sufficiently acidic to allow the conversion Tf to apoTf.

Tf-Tfr Interactions Early During Biosynthesis

At early time points of chase, no Tf bound to Tfr is detectable (Figs. 3 *A* and 5 *A*). Because at that time both Tf and most Tfr will be located in the ER (since both are fully Endo H sensitive, Fig. 3 *A*), the ER-resident Tfr apparently does not immediately acquire ligand binding capacity. apoTf will not bind to Tfr at neutral pH. After 15 min of chase, Tf binding becomes detectable (Fig. 3 *A*). To provide higher temporal resolution in the analysis of events leading to a functional Tfr, HepG2 cells were pulsed for 5 min and chased for the times indicated (Fig. 5 *A*). Tfr was analyzed under reducing and nonreducing conditions, the latter allowing the separa-

tion of the Tfr monomer and the disulfide linked Tfr homodimer (Fig. 5 *A*). Tf binding was detected at 20 min of chase, consistent with the earlier experiment (Fig. 3 *A*). The inability to observe interactions of Tfr with Tf during the earliest time points of chase is not a consequence of incomplete N-linked glycan maturation: pulse-chase experiments in the presence or absence of the glucosidase inhibitors (11) dNM or castanospermine revealed no effect on Tf-Tfr interactions (data not shown). The acquisition of Tf binding capacity coincided with covalent Tfr dimerization (Fig. 5 *A*). Formally, these results do not allow the conclusion that only the disulfide linked Tfr dimer has Tf binding capacity (since radiolabeled Tf can bind to nonradioactive Tfr synthesized during the chase) and, therefore, that the interaction of Tfr with Tf can in fact occur in the ER.

To show that Tf-Tfr interactions can take place in the ER, pulse-labeled HepG2 cells were incubated at 10°C for different periods (Fig. 5 *B*). When analyzed under nonreducing conditions, the relative amount of covalently dimerized Tfr was found to increase with time, and, in parallel, the quantity of Tf bound. Even though membrane traffic is arrested
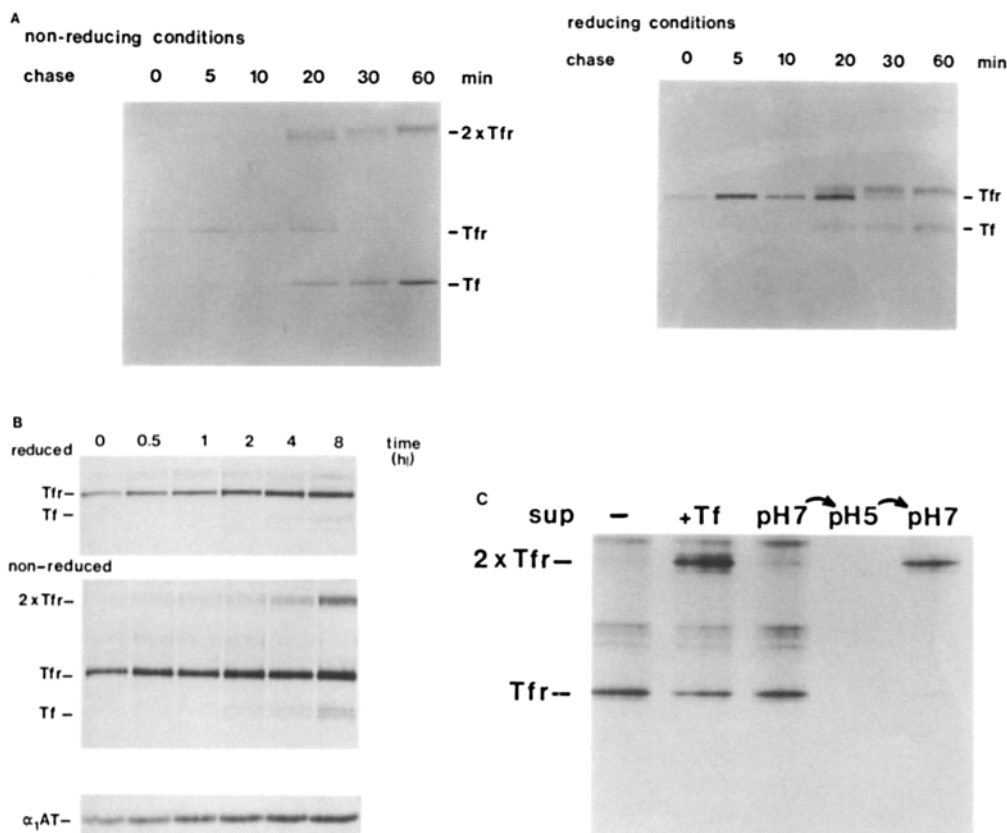

Figure 5. Interactions of Tf and Tfr early during biosynthesis. (*A*) Formation of disulfide-linked Tfr dimer early during biosynthesis. HepG2 cells were labeled for 5 min and chased for the times indicated. Tfr was immunoprecipitated and analyzed by SDS-PAGE under reducing and nonreducing conditions. The disulfide-linked Tfr homodimer (2 × *Tfr*) is observed under nonreducing conditions after 20 min of chase. Tf binding is observed as soon as the covalent Tfr dimer has been formed. The positions of the covalent Tfr dimer (2 × *Tfr*), Tfr monomer, and Tf are indicated. (*B*) Time dependency of Tf-Tfr interactions. HepG2 cells were labeled for 7.5 min and chased at 10°C for the times indicated. At 10°C all intracellular transport is inhibited as indicated by the absence of any further posttranslational modifications (see Fig. 3). Tfr was immunoprecipitated out of equal amounts of TCA-precipitable radioactivity, followed

by a sequential immunoprecipitation of α_1AT. Immunoprecipitated Tfr was analyzed under reducing and nonreducing conditions. Disulfide-linked Tfr dimers (2 × *Tfr*) as well as Tfr-bound Tf are observed after 1 h of chase at 10°C. (*C*) Disulfide-linked Tfr dimer but not the Tfr monomer has Tf binding capacity. A lysate (pH 7.4) of the erythroleukemic cell line K562 (labeled with [^{35}S]methionine) was incubated with Tf-Sepharose in the absence (lane marked −) or presence (lane marked +*Tf*) of 10 μg/ml Tf as indicated. Tfr was immunoprecipitated from the unbound fraction and analyzed by SDS-PAGE under nonreducing conditions (lanes marked −, +*Tf*, *pH 7*). The positions of Tfr monomer and the disulfide linked Tfr dimer (2 × *Tfr*) are indicated. Lanes *3-5* Tfr recovered from the unbound fraction. The Tf-Sepharose-bound fraction was exposed to pH 5.1, resulting in the formation of apoTf-Tfr complexes. Tfr was not eluted by this procedure (lane *pH 5*). The Tf-Sepharose-bound fraction was then again exposed to pH 7.4 (rightmost lane, *pH 7*). Tfr was now recovered from the eluate. The disulfide-linked Tfr homodimer is released because the apoTf-Tfr complex is not stable at pH 7.4. Note the minor fraction of Tfr monomer released.

at 10°C (note that no glycan modifications are detected for either Tf, Tfr, or α_1AT as deduced from characteristic shift in electrophoretic mobility), covalent Tfr dimerization proceeds at 10°C, and results in the generation of a functional Tfr. We conclude that this Tfr interacts with Tf in the ER.

To show directly that it is the covalent Tfr homodimer that binds Tf, a lysate of K562 cells (which express large amounts of Tfr) that had been labeled with [^{35}S]methionine for 60 min, was passed over a Tf-Sepharose column. Tfr was immunoprecipitated from the flow-through of this column and analyzed by nonreducing SDS-PAGE (Fig. 5 C). The disulfide-linked Tfr homodimer was found to bind to Tf-Sepharose and could only be maintained in the flow-through when the lysate was supplemented by excess, free Tf before passage over Tf-Sepharose. Elution of the Tf-Sepharose–retained material at pH 5.1 did not result in the release of Tfr. At pH 5.1 Tf is converted to apoTf (Fe^{3+} is released) but the apoTf–Tfr complex dissociates only when exposed subsequently to neutral pH (7, 17). Indeed, subsequent exposure of the pH 5.1–treated, bound fraction to pH 7.4 resulted in the release of the disulfide-linked Tfr homodimer. Only a minor fraction of Tfr monomer (or nondisulfide-linked Tfr dimer; these two cannot be distinguished by this criterion) was observed (Fig. 5 C, last lane).

Discussion

The intracellular interactions of Tf and Tfr synthesized by the human hepatoma cell line HepG2 that we have documented, are depicted schematically in Fig. 6. Early during biosynthesis the Tfr monomer is converted to a disulfide-linked homodimer (Fig. 5 A). Covalent dimerization can proceed even at 10°C, albeit slowly, in the ER (Fig. 5 B). The process of covalent dimerization is largely completed in the ER (Fig. 5, A and B), but in any case, before arrival in the trans-Golgi apparatus. The formation of this disulfide-linked Tfr may proceed through a nondisulphide-linked Tfr dimer intermediate. The small quantity of Tfr monomer recovered in the experiment shown in Fig. 5 C may in fact derive from such noncovalent dimers. Tfr obtains Tf binding capacity upon dimerization (Fig. 5 C). The formation of disulfide-linked Tfr dimers is not a prerequisite for transport out of the ER because elimination of the two interchain disulfide bridges by site-directed mutagenesis does not eliminate surface expression or Tf binding properties of Tfr (15). However, it is possible that Tfr monomers do not leave the ER and Tfr can only do so through the formation of (covalent or noncovalent) dimers (see also reference 12).

The Tf in the Tf-Tfr complex must contain Fe^{3+}, which it

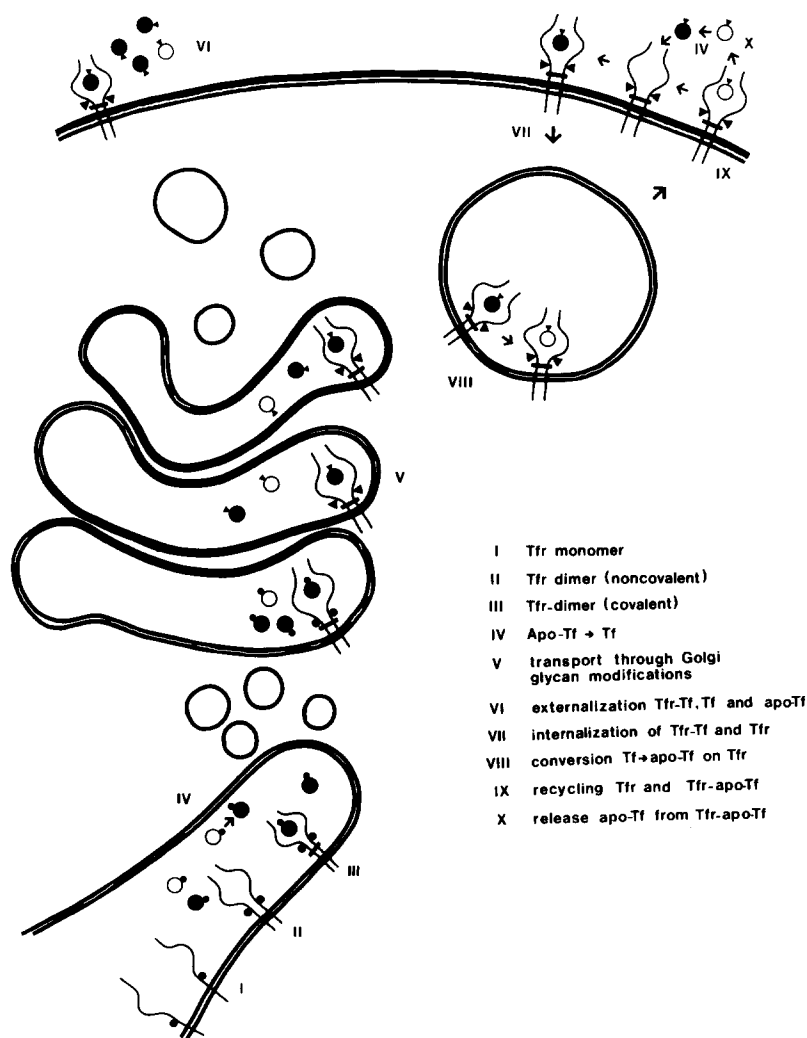| I	Tfr monomer
II	Tfr dimer (noncovalent)
III	Tfr-dimer (covalent)
IV	Apo-Tf → Tf
V	transport through Golgi glycan modifications
VI	externalization Tfr-Tf,Tf and apoTf
VII	internalization of Tfr-Tf and Tfr
VIII	conversion Tf→apo-Tf on Tfr
IX	recycling Tfr and Tfr-apoTf
X	release apo-Tf from Tfr-apoTf

Figure 6. Schematic representation of the Tf and Tfr interactions in HepG2 cells. The Tfr monomer, as initially synthesized, is converted to a disulfide-linked Tfr dimer. This may go through a nondisulfide linked Tfr dimer as an intermediate. The Tfr dimer has Tf binding activity in the ER, whereas the Tfr monomer has not. The resulting Tf-Tfr complex is transported through the Golgi apparatus and the TGR. The TGR has a pH >5.5. Direct transfer of Tf-Tfr and Tfr from the secretory pathway (trans-Golgi) to the endocytic route (from IV to VIII) is a possibility not excluded by our experiments. When the Tf-Tfr complex arrives at the cell surface, the Tf-Tfr is internalized and arrives in an (acidic) endosome where Tf releases its Fe^{3+} and is converted to apoTf. The apoTf-Tfr complex (stable at acidic pH) recycles back to the cell surface. The apoTf-Tfr complex is unstable at neutral pH, dissociates, and Tfr can bind a new Tf molecule from the surrounding medium and starts a new Tf cycle.

will have obtained in the ER (Fig. 5 *B*). Not all Tf binds to Tfr, because Tf is synthesized in molar excess (Fig. 3 *B*; Tf contains 9 [21, 37], Tfr contains 11 methionines [23, 32]). In addition, not all newly synthesized Tf may have bound Fe^{3+} and thus be able to bind to Tfr. The Tf–Tfr complex is transported through the Golgi and the *trans*-Golgi reticulum (TGR). The pH in the TGR is not sufficiently acidic to convert free Tf to apoTf (Fig. 4 *B*) and must therefore be higher than pH 5.5 (7, 17) (unless the residence time of Tf in the TGR would be in the order of 1.5 min, the reported half-time of conversion of Tf to apoTf at pH 5 [7]). Thus, the local conditions are probably compatible with safe passage of Tf–Tfr through the TGR.

The experiments in Fig. 4 show that the Tf–Tfr complex is rapidly delivered from the TGR to a compartment of low pH, the target of action of chloroquine. The exact route by which this acidic compartment is reached cannot be established with certainty from our experimental data. It is possible that the Tf–Tfr complex is deposited at the cell surface, from which it is rapidly internalized via the usual endocytic pathway (7, 17). Alternatively, the Tf–Tfr complex may be delivered directly to the endocytic pathway, and enter the transferrin cycle intracellularly. The kinetics of this process as observed experimentally are too rapid to allow a clear distinction between these two possibilities. For molecules like the mannose-6-phosphate receptor (19) and the major histocompatibility complex class II antigens (26), a direct connection between the TGR and the endocytic route has been shown.

Because Tfr and Tf can interact intracellularly in the course of biosynthesis, this possibility probably also exists for other ligand–receptor combinations synthesized by one and the same cell. In the case of a growth factor and its receptor, such interactions can result in transport to the cell surface and delivery of a growth signal. Whether a growth signal can already be delivered after binding of a growth factor to its receptor in the ER or Golgi is not known. There are several ways by which premature interaction of receptors with their ligands could be prevented. For example, covalent modifications (e.g., disulfide bonding, glycan modifications, phosphorylations) required for full ligand binding potential may take place at a time and location where ligand and receptor are no longer available in the same compartment. The presence of cofactors (e.g., divalent cations, optimal pH for binding) may likewise be a controlling factor.

Coexpression of EGF or $TGF\alpha$ and the EGF-receptor has been observed in a number of tumors and cell lines (8) and a similar situation may apply to other growth factor–growth factor receptor combinations. Their intracellular interactions, which could ultimately contribute to tumor growth, might go along lines parallel to those outlined here for Tf–Tfr.

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