Release of the Soluble Transferrin Receptor Is Directly Regulated by Binding of Its Ligand Ferritransferrin*

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The human transferrin receptor (TfR) is shed by an integral metalloprotease releasing a soluble form (sTfR) into serum. The sTfR reflects the iron demand of the body and is postulated as a regulator of iron homeostasis via binding to the hereditary hemochromatosis protein HFE. To study the role of transferrin in this process, we investigated TfR shedding in HL60 cells and TfR-deficient Chinese hamster ovary cells transfected with human TfR. Independent of TfR expression, sTfR release decreases with increasing ferritransferrin concentrations, whereas apo-transferrin exhibits no inhibitory effect. To investigate the underlying mechanism, we generated several TfR mutants with different binding affinities for transferrin. Shedding of TfR mutants in transfected cells correlates exactly with their binding affinity, implying that the effect of ferritransferrin on TfR shedding is mediated by a direct molecular interaction. Analysis of sTfR release from purified microsomal membranes revealed that the regulation is independent from intracellular trafficking or cellular signaling events. Our results clearly demonstrated that sTfR does not only reflect the iron demand of the cells but also the iron availability in the bloodstream, mirrored by iron saturation of transferrin, corroborating the important potential function of sTfR as a regulator of iron homeostasis.

The transferrin receptor (TfR)3 is a homodimeric type II membrane protein that mediates iron uptake into the cell. Each subunit possesses one binding site for the iron carrier protein transferrin. The complex of ferritransferrin (ferri-Tf) and TfR is transported into the cell by clathrin-mediated endocytosis. Inside the acidic environment of the endosome, iron dissociates, and the complex of apo-transferrin and TfR recycles back to the plasma membrane, where apo-transferrin is released, and TfR is presented for a new uptake cycle. The TfR consists of a small cytoplasmic domain, a single-pass transmembrane region for each subunit, and a large butterfly-shaped ectodomain, which is kept by a stalk at a distance of 2.9 nm from the plasma membrane (1). The crystal structure of the extracellular domain revealed that each homodimer has three structurally distinct domains: a protease-like domain following the stalk, a helical domain mediating dimer contact, and an apical domain oriented toward the outside (2).

Early approaches to identify the transferrin binding site on the TfR were based on chimeras of human TfR and the chicken counterpart that does not bind human transferrin. Mapping of the receptor-ligand interaction revealed the helical domain of the TfR as binding site (3). Another mutational study with soluble chimeras of TfR and alkaline phosphatase identified a critical RGD sequence for transferrin binding within the helical domain (4). This observation is consistent with a newer extensive mutational analysis wherein further important residues for transferrin binding were identified (5). The combined data from structural analyses with x-ray hydroxyl radical footprinting (6) and a subnanometer resolution by cryo-electron microscopy (7), as well as from the crystal structures of ferri-Tf (8, 9) and the TfR ectodomain (2), demonstrated that the C-lobe of transferrin abuts against the helical domain, whereas the N-lobe is sandwiched between the membrane and the TfR ectodomain (7), indicating a binding to the stalk region of the TfR.

Besides the membrane-associated cellular TfR, a soluble form (sTfR) exists in human serum. It is released by proteolytic cleavage of the TfR C-terminal of Arg-100 within the stalk (1, 10). The shedding process is primarily mediated by an integral membrane metalloprotease sensitive to the inhibitor TAPI-2 (11). The protease probably belongs to the disintegrin and metalloprotease family (ADAM) (11). Nevertheless, other proteases are also involved in cleavage at alternative sites (12, 13).

In mammalian organisms, iron is required for the function of various proteins in fundamental biochemical reactions. Since iron also harbors a toxic potential, uptake into the body must be strictly controlled to preserve iron homeostasis. The divalent metal transporter DMT-1 mediates apical dietary iron absorption in the duodenum via villus cells. On the basolateral surface, iron is exported by the iron transporter ferroportin (also named iron-regulated protein IREG-1) and then bound to transferrin. The mechanism of how the enterocytes sense the iron demand of the body, however, is unknown. Three soluble proteins or peptides are believed to be involved in the basolateral regulation of iron export from duodenal enterocytes to the serum, namely sTfR, transferrin, and hepcidin. The concentrations of these molecules in serum change depending on the iron status of the body. It remains unclear how they transfer the signal to the enterocyte. Recently, it has been shown that hepcidin binds to ferroportin and induces its internalization (14).

The sTfR mirrors the availability of functional iron independent of the iron stores in the body (15–17). In thalassemia major, the patients exhibit high sTfR levels (18) as well as high duodenal iron uptake, although the iron stores of the body are filled (19). Unlike other iron markers, sTfR is not affected by chronic inflammation or infection. The average sTfR level in normal subjects is 5 mg/liter and can vary from 1/8 to 20-fold of average if iron demand is changed (17). Since erythroid precursor cells contain 80% of total body TfR mass, the sTfR is directly

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3 The abbreviations used are: TfR, transferrin receptor; sTfR, soluble TfR; ferri-Tf, ferritransferrin; FITC, fluorescein isothiocyanate; HFE, classic hemochromatosis protein 1; NHS, N-hydroxysuccinimidyld-4-azidosalicylic acid; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; WT, wild type.
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correlated with the total mass of erythroid precursors (18). Nevertheless, not only erythroid precursors but all cells of the body are involved in sTfR production, in particular rapidly dividing cells and cells of the liver and placenta. Thus, the sTfR is more likely a marker for tissue iron deficiency and not only a determinant of erythroid precursor mass. Iron deficiency leads to an sTfR increase proportional to the severity of iron deficit (20).

New insights into the regulation of iron metabolism are gained from a common disease connected to iron homeostasis, the hereditary hemochromatosis type I. A mutation in the hemochromatosis protein (HFE) results in severe body iron overload and progressive organ failure. HFE has a direct influence on the iron concentration in reticuloendothelial cells (21). Furthermore, studies using the macrophage cell line THP-1 and the intestinal cell line HT-29 reveal an effect of HFE on iron export rather than iron uptake (22, 23), suggesting an inhibitory effect of HFE on ferroportin function. Other authors propose a function of HFE in the regulation of hepcidin expression in the liver, which in turn could regulate iron export from the gut (reviewed in Ref. 24).

It has been observed that sTfR as well as TfR bind to HFE in tissues and cell lines (25–27), suggesting that the inhibitory activity of HFE on iron export is regulated by sTfR. Since sTfR reflects the iron demand of the body and has the ability to bind HFE, it is discussed in recent years as the erythroid regulator of iron homeostasis (24, 28). A regulation of TfR shedding by extracellular compounds is, however, unknown to date. Since iron saturation of transferrin reflects iron availability, the release of sTfR from the cell surface may be controlled by transferrin. Therefore, in the present study, we analyzed the effect of transferrin on TfR shedding.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—Mouse monoclonal antibody OKT9 directed against the extracellular domain of TfR was prepared from a hybridoma cell line as described previously (29). Polyclonal rabbit antibody pAB063 was generated by immunization with purified human placental TfR (in cooperation with R. Gessner, Charité, Campus Virchow-Klinikum, Berlin, Germany). Horseradish peroxidase-labeled anti-mouse and anti-rabbit antibodies were obtained from Dako A/S (Glostrup, Denmark), FITC-labeled goat anti-mouse antibody was from Alexis (Grüneberg, Germany), ferri-Tf was from Sigma, blasticidin S and 3% bovine serum albumin in PBS. The cells were homogenized by using a Dounce homogenizer 30 times and differentially centrifuged at 5000 times g for 20 min. The supernatant containing microsomal membranes was washed once with PBS, 0.1% EDTA. The membrane pellet was resuspended in PBS to a final concentration of 1 mg/ml total protein, and 35-μl aliquots were incubated for 20,800 × g, 4 °C, 20 min) to remove cellular debris. Bound TfR (or sTfR) was quantitated in a colorimetric reaction using tetramethylbenzidine after detection with anti-TfR polyclonal antibody pAB063 (1:1000 in PBS, 0.05% Tween 20) and peroxidase-labeled swine anti-rabbit IgG (1:2000 in PBS, 0.05% Tween 20). The purification of placental TfR, which served as internal standard for the quantitation of sTfR, and colorimetric reaction were performed as described (30).

Detection of sTfR Release from Membranes—After removal of the medium, transfected TRVb cells were placed on ice, washed twice with 10 ml of PBS, and then incubated for 15 min with 5 ml of 1:10 diluted PBS. The cells were homogenized by using a Dounce homogenizer 30 times and differentially centrifuged at 500 × g for 15 min followed by 2600 × g for 15 min and finally 100,000 × g for 30 min. The pellet from the last centrifugation step containing microsomal membranes was washed once in 1 ml of PBS and centrifuged at 20,000 × g for 20 min. The membrane pellet was resuspended in PBS to a final concentration of 1 mg/ml total protein, and 35-μl aliquots were incubated at 37 °C for 12 h in the presence or absence of 10 μg/ml of ferri-Tf. The samples were then centrifuged at 20,000 × g for 20 min at 4 °C and sTfR in the supernatants analyzed in the TfR-specific ELISA as described above.

Depletion of sTfR from Ferri-Tf Solution—OKT9 antibody (650 μg) was covalently bound to NHS-Sepharose as described by the manufacturer. Ferri-Tf solution (4 mg/ml containing 46 ng/ml sTfR as measured in TfR-ELISA) was incubated overnight with OKT9-NHS-Sepharose. After removal of the Sepharose, neither OKT9 (tested by ELISA with rabbit anti-mouse antibody) nor sTfR (tested by TfR-ELISA) was detectable (detection limit for both 0.08 ng/ml of ferri-Tf).

Detection of TfR Located at the Cell Surface of Transfected TRVb Cells—After washing once with PBS, transfected TRVb cells were detached from the plate by PBS, 0.1% EDTA. The suspended cells (3 × 10^8/sample) were blocked with PBS, 1% goat serum and incubated with 1 μg/ml IgG1 (isotype control) or 1 μg/ml OKT9. After washing three times, the cells were stained with FITC-labeled goat anti-mouse IgG (1:50 in blocking solution) and washed again. Each incubation step was performed for 30 min at 4 °C. Finally, cells were resuspended in 150 μl of PBS and TfR on the cell surface determined by flow cytometry (FACS-calibur, BD Biosciences) and CellQuest software.
Binding of Ferri-Tf to Transfected TRVb Cells—After washing, detaching, and blocking as described above, $3 \times 10^6$ transfected TRVb cells were incubated with 4 μg/ml ferri-Tf-FITC (generated by labeling ferri-Tf using the FluoroReporter FITC protein labeling kit from Molecular Probes, Leiden, Netherlands) for 30 min at 4 °C. Untransfected TRVb cells served as control. After washing three times, cells were resuspended, and bound ferri-Tf was quantitated by flow cytometry as described above.

Determination of the Equilibrium Dissociation Constants of the Ferri-Tf–TfR Complex—Equilibrium dissociation constants for the complex of TfR mutants and ferri-Tf were determined using Liliom plot analyses (31) based on data from a receptor-ligand ELISA (32). Briefly, 96-well microplates were coated with 500 ng of ferri-Tf in PBS for 90 min, blocked with PBS, 10% fetal calf serum, 3% bovine serum albumin for 30 min, and incubated for 2.5 h with lysates of TfR-transfected TRVb cells in concentrations between 55 pm and 55 nm (determined using the TfR-specific ELISA). Bound TfR was detected as described for the TfR-specific ELISA. The absorbance (recorded at 450 nm) at maximal binding was derived from a plot of the TfR concentration versus absorbance by curve fitting. $K_D$ values were determined as the reciprocal value of the slope in a Liliom plot ($\text{absorbance} / c$ versus $1/(1 - i)$, where $c$ is the TfR concentration and $i$ the quotient of the absorbance at a given TfR concentration and the absorbance at maximal binding) (31).

RESULTS

Influence of Ferri-Tf on TfR Shedding—Since the sTfR concentration in human serum mirrors the iron demand of the body, it can be expected that TfR shedding is regulated by proteins that reflect the actual iron status. The concentration of iron-loaded transferrin (ferri-Tf) is high when available body iron is abundant. Since the sTfR concentration is reduced in this case, we raised the hypothesis that ferri-Tf has an inhibitory effect on TfR shedding.

To test this hypothesis, we measured sTfR release into serum-free culture medium from HL60 cells, a human promyelocytic cell line generally used to study TfR shedding (11, 33, 34). Since we found that commercially available ferri-Tf (Sigma) is contaminated with sTfR (12 ng/mg of ferri-Tf), which interferes with the measurement, we first completely removed the sTfR from ferri-Tf by treatment with OKT9-NHS-Sepharose. Using an ELISA developed in our laboratory (11), we specifically determined both the amount of released sTfR and the amount of cellular TfR. To depict alterations in sTfR release independent of possible changes in cellular TfR during ferri-Tf incubation, we calculated the amount of released sTfR as a percentage of cellular TfR. Our results demonstrate that with increasing sTfR-free ferri-Tf concentrations, TfR shedding significantly ($p$ values less than 0.05, in most cases less than 0.01) decreases gradually, independent of the cellular TfR expression (Fig. 1A). Moreover, within the time interval of the experiment, the expression of cellular TfR is virtually unchanged. Only a slight variation of extracellular iron supply, we transfected the TRVb cells with human HL60 cells (13). Cells transfected with human TfR were analyzed they exhibit the same protease inhibition pattern on TfR shedding as H68.4 and OKT9 directed against the intracellular and extracellular domain of TfR, respectively, and shown to be clearly expressed in sTfR release from 106 cells either compared with untreated cells (A, C, and D) or compared with 106 cells (B) tested with a paired Student’s $t$ test ($^*$, $p < 0.05$; $^{**}$, $p < 0.01$; $^{***}$, $p < 0.005$).

Inhibition of sTfR release is reached at 200–400 μg/ml for 106 and above 1000 μg/ml for 107 cells. Thus, the concentration for maximal inhibition shifted, as expected, to higher concentrations. Since significantly higher cell concentrations than 107 cells/ml cannot be applied in the chosen experimental design, an exact titration of cell number versus concentration cannot be performed. Nevertheless, the experiment demonstrated that the concentration interval, in which inhibition is subject to significant changes, is in the physiological range of ferri-Tf (150–500 μg/ml). The results are corroborated by the observation that treatment with the Fe3+ chelator desferal counters the effect of ferri-Tf on TfR shedding (Fig. 1D), indicating that iron-free apo-transferrin, which has a low affinity to TfR, cannot inhibit TfR release.

For further studies, we used transfected TRVb cells, which enabled us to investigate TfR mutants. TRVb cells release high amounts of sTfR (13.3 fg/ml/cell as compared with 5.8 fg/ml/cell in HL60). Furthermore, they exhibit the same protease inhibition pattern on TfR shedding as human HL60 cells (13). Cells transfected with human TfR were analyzed by SDS-PAGE of the lysates and Western blotting with the anti-TfR antibodies H68.4 and OKT9 directed against the intracellular and extracellular domain of TfR, respectively, and shown to be clearly expressed with the expected molecular weight. The correct localization was proven by immunofluorescence studies. Permeabilized and non-permeabilized cells were stained with OKT9 and rhodamine-conjugated goat anti-mouse antibody. The receptor was exclusively localized on the cell surface and inside endosomes as expected (not shown).

To exclude a feedback effect on intracellular TfR translation during variation of extracellular iron supply, we transfected the TRVb cells with human TfR lacking all the iron-responsive elements 3’ to the open read-
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ing frame. Analogous to HL60 cells, TRV shedding in transfected TRVb cells significantly (p values less than 0.005) decreases with increasing ferri-Tf concentrations (Fig. 1C) and increases with decreased transferrin saturation generated by adding desferal (Fig. 1D). The observed effect is clearly independent of the cellular TR expression, which exhibits only an unsteady variation of maximal 6% at the different ferri-Tf concentrations. As in HL60 cells (Fig. 1A), maximal inhibition (25–31%) is attained at ferri-Tf concentrations of 200 ng/ml. The lower shedding rate per TR (Fig. 1, compare A with C) is a result of 11 times higher TR expression in transfected TRVb cells and not of a decrease in the amount of released sTFR.

Mammalian cells usually shed a number of different cell surface proteins. To show that the effect mediated by ferri-Tf is specific for the TR, we additionally quantified the shedding of L-selectin in HL60 cells by an analogous ELISA. The amount of released soluble L-selectin unsteadily varied in a narrow range of 0.97–1.21 ng/ml for the different ferri-Tf concentrations with virtually no correlation (r = 0.27), demonstrating that ferri-Tf does not affect shedding processes in general.

**TFR Mutants with Decreased Affinity to Transferrin—**To solve the question of whether the effect of ferri-Tf on TR shedding is mediated by direct binding to the TR or indirectly by binding to another component that mediates protease inhibition by intracellular signal transduction events or adapter proteins, we generated TRR mutants with different binding affinities for transferrin. Results from other authors show that mutations within the RGD sequence in its extracellular domain lead to altered dissociation constants for the ferri-Tf-TRR complex (see “Discussion”). Therefore, we introduced the conservative mutations R646K, G647A, and D648E into full-length TRR.

Expression and localization of mutant TRR was analyzed by immunofluorescence as described above for wild-type (WT) TRR and found to be identical (not shown). To quantify the cell surface expression of WT-TRR and TRR mutants on individual cells, TRR was labeled with OKT9 and FITC-conjugated goat anti-mouse antibody and analyzed by flow cytometry (Fig. 2, left panel). Dependent on the mutant, the percentage of cells that express TRR varied from 56% for WT to 73% for R646K (Fig. 2, left panel, right peaks). Cells not expressing TRR do not interfere since TRVb cells do not express endogenous TRR and are therefore unable to bind transferrin nor to release sTFR. The cells expressing WT-TRR, G647A, and D648E exhibit similar levels of expression on the cell surface; the R646K mutant possesses fewer TRR per cell (Fig. 2B, left panel); however, this mutant has the highest number of cells that express TRR. Thus, the mutants behave like WT-TRR with respect to localization and distribution and are therefore suitable for the further studies. Differences in the total amount of TRR expression were taken into account by calculating the ratio of released sTFR and cellular TRR.

To demonstrate that the mutants indeed exhibit different binding affinities for transferrin, the cells were incubated with ferri-Tf-FITC and analyzed by flow cytometry. No ferri-Tf binding is detectable in transfected TRVb cells and neither does the binding of ferri-Tf to cells expressing R646K (ratio of 4.1) nor detectable when transfected with G647A (ratio of 584).

After measuring the binding of ferri-Tf to transfected TRVb cells, we determined the affinity constants of the binding partners in vitro to confirm the results and to quantitate the binding, permitting a clear comparison with the amount of sTFR release. The dissociation constants were determined by a Liliom plot using binding data derived from a receptor-ligand ELISA. This analysis yields KD values with ferri-Tf binding ratios in fluorescence-activated cell sorter experiments. Due to their gradual reduction in ferri-Tf binding, the TRR mutants are optimal tools to examine the influence of transferrin binding on TRR shedding.

**sTFR Release from Transfected Cells—**Transfected TRVb cells were cultured in ferri-Tf-containing medium. TRR shedding from these cells was determined using the TRR-specific ELISA. The amount of sTFR in the culture supernatant increases in a time-dependent manner for all cell lines, but the extent of the increase differs (Fig. 3A). Nevertheless,
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FIGURE 3. Release of sTfR from transfected TRVb cells. Cells (5 × 10⁵/ml) were seeded and cultured for 1–4 days (A and B). The cells were counted, the cell supernatants were collected, and the concentration of sTfR and cellular TfR was analyzed in a TfR-specific ELISA. The plot in A shows the time course of sTfR release from cells expressing WT-TfR (squares) and the TfR mutants R646K (circles), G647A (triangles), and D648E (diamonds), and the plot in B shows the proliferation rate of all these cell lines. Data represent mean values ± S.E. of five independent experiments performed in triplicate. C, cells (1 × 10⁵/ml) were incubated for 12 h in ferri-Tf-containing medium. Dark gray bars represent the sTfR release from cells expressing WT-TfR and mutated TfR as percentage of cellular TfR. Light gray bars show the dissociation constants (Kₕ) of the corresponding ferri-Tf-TfR complexes. Data (± S.E.) were derived from the analyses of Liiom plots from three independent experiments. The equilibrium response data were performed in triplicate for each concentration.

FIGURE 4. Effect of ferri-Tf on sTfR release from purified membranes. Purified membranes of transfected TRVb cells were incubated for 12 h at 37°C in the presence or absence of ferri-Tf. The release of sTfR was measured in three independent experiments in a TfR-specific ELISA. A, influence of ferri-Tf on sTfR release from purified microsomal membranes derived from TRVb cells transfected with WT-TfR. B, dark gray bars, the relative inhibitory effect of ferri-Tf on sTfR release from purified microsomal membranes containing WT-TfR (corresponding to the relative difference of the bars in panel A) and the different TfR mutants. Light gray bars, association constants (Kₖ) for the ferri-Tf-TfR complex (derived from Fig. 3C for better comparison).

Advantage of excluding trafficking or signal transduction events (11). Purified membrane fractions of TfR expressing TRVb cells were incubated in the presence or absence of ferri-Tf. Both TfR and the shedding protease were located in the purified membranes and were still active under the assay conditions. Released sTfR was quantitated in the supernatant of the membranes by ELISA. The addition of ferri-Tf clearly reduces the amount of released WT-sTfR up to 46% (Fig. 4A). Thus, the inhibitory effect of ferri-Tf cannot be attributed to signal transduction in the cytosol or relocalization of the TfR in vicinity to the shedding protease but rather results from a direct inhibition. If the direct effect is a consequence of an interaction of ferri-Tf with the shedding protease, the inhibition of TfR shedding should not be affected in the TfR mutants. In contrast, if ferri-Tf competes for the binding site or induces allosteric inhibition, the blocking of TfR shedding by ferri-Tf should be decreased in the low affinity mutants. In Fig. 4B, the higher percentage represents a stronger inhibitory effect by ferri-Tf. Inhibition of TfR shedding in microsomal membranes is slightly reduced from 46% for WT-TfR to 38% for D648E and 34% for R646K, whereas shedding of the low affinity mutant G647A is clearly reduced to 23%. These values clearly mirror the corresponding binding affinities for ferri-Tf (inhibition is proportional to the pKₖ). Thus, our results demonstrated that binding of ferri-Tf to the TfR down-regulates TfR shedding by blocking the binding of the shedding protease or by preventing cleavage due to induced structure alterations. In conclusion, an increased intracellular iron demand was manifested in elevated sTfR release due to constitutive shedding of an augmented amount of TfR, whereas extracellular iron availability mirrored by ferri-Tf antagonized TfR shedding.

DISCUSSION

In the present study, we investigated the influence of transferrin on TfR shedding. Due to its altered concentration in diseases of iron metabolism and its ability to bind HFE, sTfR is, together with hepcidin, one of the most discussed proteins involved in systemic iron regulation (24, 28).

Transferrin saturation represents a reliable indicator for instantaneous iron availability of the organism; however, as known to date, it does not influence iron absorbance from the gut (22). We demonstrated that increasing ferri-Tf concentrations results in decreased TfR shedding rates in both HL60 and TRVb cells transfected with WT-TfR, whereas the iron chelator desferal has the opposite effect. The concentration of...
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ferri-Tf that blocks half of the sTfR release was in a range corresponding to a typical transferrin saturation in the transition from iron deficit to normal iron status. Our in vitro data correspond very well with in vivo analyses newly published by Brandao et al. (35), who find an sTfR decrease in human serum with increasing transferrin saturation as long as the saturation is below 25%. Maes et al. (36) showed that sTfR and iron inversely correlate to each other in humans. The inhibitory effect of ferri-Tf on TfR shedding is based on a mechanism independent of TfR transcription and translation. Under the applied conditions, varying ferri-Tf concentrations had, if any, a marginal effect on TfR expression in HL60 cells and, as expected, no effect in transfected TRVb cells due to the lack of iron-responsive elements. These observations are in agreement with earlier studies of Baynes (37), who showed that TfR expression first changes after 24 h of incubation, whereas no difference was detectable after 6 h. In the same study, the author showed that an increased ferri-Tf level decreases the ratio of sTfR release and TfR detectable after 6 h. In the same study, the author showed that an increased ferri-Tf level decreases the ratio of sTfR release and TfR expression, which is consistent with our data. In HL60 cells, the author determined a decrease in sTfR release up to 59% after a 6-h incubation with 1320 pmol/liter iron as diferric transferrin (53 μg/ml ferri-Tf). In contrast, Kohgo et al. (38) and Chitambar et al. (39) assumed an activating effect of ferri-Tf on TfR shedding or no influence, respectively. As proposed by Baynes (37), these findings could be a result of sTfR contamination of the ferri-Tf preparation. Our data supported this assumption as we observed an apparent high sTfR release exerted by contaminated ferri-Tf. We therefore depleted the sTfR from commercially available ferri-Tf by antibody purification.

To address the question of how transferrin mediates this effect, we generated TfR mutants with different affinities for ferri-Tf. Earlier studies showed that the exchange of glycine 647 to alanine (G647A) within the RGD motif results in a prominently decreased binding affinity of ferri-Tf for recombinant soluble forms of TfR expressed in insect cells (5, 40). In contrast to the glycine residue, the influence of arginine and aspartate was only analyzed in chimeras in which the extracellular domain of the TfR was fused to alkaline phosphatase, resulting in a soluble reporter protein (4). In these chimeras, an R646K mutation led to reduced transferrin binding, whereas a D648E mutation had only a weak influence. In agreement with these studies, our results likewise showed that mutation of the amino acids Arg and Gly within the RGD sequence results in a moderate and strong decrease in affinity for ferri-Tf, respectively, whereas the influence of the Asp residue was low but significant. The dissociation constant of 3160 nM for the mutant G647A is in correlation with other investigations (KD = 2345 nM (5)). In contrast to previous studies by other authors, we determined dissociation constants for full-length TfR that was expressed in mammalian cells. Furthermore, we determined for the first time concrete dissociation constants for Arg and Asp mutations within the RGD sequence of TfR.

By use of transfected cells expressing these different TfR mutants, we confirmed the data obtained by varying the ferri-Tf concentration. The shedding activity of these cells showed a strong inverse correlation with the binding affinity of the TfR mutants for ferri-Tf. Thus, inhibition of TfR shedding by ferri-Tf depended on direct binding of ferri-Tf to its receptor. Since our experiments on purified microsomal membranes revealed that the influence of ferri-Tf on TfR shedding is the same as in living cells, the inhibitory effect of ferri-Tf is not a result of either signal transduction events or enhanced endocytosis of TfR, as supposed by Baynes (37). Moreover, other causes, such as changes in the cellular distribution of the receptor in relation to the shedding protease, are also excluded, indicating instead a direct effect on proteolytic cleavage, most probably by blocking the cleavage site of the TfR. This is corroborated by new structural analyses of the Tf-TfR-complex, showing that the N-lobe of ferri-Tf is lodged between the ectodomain of the TfR and the membrane (7), representing the stalk region. This allows ferri-Tf to act as a competitive inhibitor for the binding of the protease to the TfR, although it cannot be finally excluded that the binding of ferri-Tf only changes the structure of the receptor, which in turn prevents binding of the protease or, if not, at least cleavage. To clarify this, purification and final identification of the protease would be helpful; however, it has already been shown that at least two proteases are involved in TfR shedding and that the proteases lose their activity when removed from the membrane (11, 13). Moreover, the membrane-mediated lateral positioning of protease and substrate is important for recognizing the correct cleavage site (41).

In the last decade, it has become obvious that shedding of membrane proteins is an important process including such different proteins as membrane-anchored growth factors and precursors of cytokines, receptors, ectoenzymes, cell adhesion molecules, and the Alzheimer precursor protein. Most of the proteases identified to be involved in shedding processes are metalloproteases, but proteases of other classes are also described (reviewed in Refs. 42–45). Ectodomain shedding is a widely branched network of competitive processes, i.e. one type of protein can be cleaved by different proteases, or vice versa, one protease can cleave several different proteins. To date, it is unclear how shedding is regulated. The size and composition of the stalk as well as the activation
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of the protease and its localization with respect to the substrate are discussed as static and dynamic conditions (13, 46–51). Our finding that ferri-Tf inhibits shedding of the TIR demonstrated that the binding of an extracellular molecule may be a general mechanism to regulate ectodomain shedding by environmental parameters.

In addition to general mechanisms, the elucidation of ferri-Tf-dependent regulation of TIR shedding sheds new light on iron homeostasis. To date, it is unclear how iron homeostasis is ensured. The sTIR level is closely related to erythroid TIR turnover, and the prime determinants of the sTIR concentration are cellular iron demand and erythropoiesis rate (18, 20). Furthermore, our studies demonstrate that sTIR does not only reflect the sum of cellular iron demand but simultaneously reflects the instantaneous iron availability of the whole body mirrored by the iron saturation of transferrin in the bloodstream, as depicted in the model in Fig. 5 (upper panel). Thus, the organism has the ability to act at an early stage to an imbalance between iron demand and iron availability. Decreasing iron saturation of transferrin (like in iron deficiency) and/or increasing cellular iron demand cause enhanced TIR shedding. In the bloodstream, released sTIR can bind to circulating ferri-Tf (Fig. 5, middle panel). Indeed, in the case of normal transferrin saturation, 95 ± 3% of sTIR can be coprecipitated with antibodies against transferrin (18). An analysis of the equilibrium between free sTIR and sTIR complexed with ferri-Tf demonstrates, however, that the concentration of free sTIR increases drastically when transferrin saturation drops below 10% (Fig. 5, lower panel). Assuming a K-D of 7 nm for the dissociation of sTIR from ferri-Tf, a 5-fold elevated sTIR concentration of 25 mg/liter due to iron deficit and low transferrin saturation (10%) results in a concentration of 1000 pm free sTIR in the bloodstream, a value 20-fold greater than under normal conditions (50 pm free sTIR in the case of 35% transferrin saturation and 5 mg/liter sTIR). The absolute free sTIR concentration and the dynamics of its variation are in the range for typical hormones like insulin (36–600 pm) and aldosterone (180–790 pm) (52).

Our data are consistent with the hypotheses formulated by Townsend and Drakesmith (28) and Cazzola et al. (53) and that free sTIR levels in the blood may activate iron export from reticuloendothelial cells or duodenal enterocytes, probably by an interaction with HFE in these cells. Our experiments give a hint about how sTIR can represent both the cellular iron demand and systemic iron availability. Indeed, Cook et al. (54) found a slight correlation between sTIR concentration and iron absorption in human. The correlation was aborted when persons with iron deficiency (ferritin <15 μg/ml) were excluded; however, this in turn means that in particular under iron deficiency conditions, sTIR may play an important role supporting our observations. With the described properties derived from our data, the sTIR completely fulfills the requirements for an erythroid regulator as formulated by Finch (55). It is effective when the total cellular iron demand of the body is greater than the compensative capability of the storage regulator to release iron from stores, in the case of the study of Cook et al. (54) under iron deficiency conditions. The model of sTIR as erythroid regulator is in line with increased iron resorption observed in β-thalassemia. In this disease, the number of reticulocytes is elevated (56), and thus, sTIR release is increased. This model is also supported by the observation that a lack of transferrin expression in humans and mice causes uncontrolled iron absorption from the gut, whereas repeated injections of transferrin in hypotransferrinemic animals results in reduced intestinal iron resorption (57). This can be explained by a drastic increase in free sTIR and by complexing free sTIR, respectively. Interestingly, plasma transfused to healthy mice does not affect iron absorption (58), indicating that the erythropoietic regulator must possess a short half-life or be neutralized as proposed by Hentze et al. (59). Our model suggests that after plasma transfusion to normal mice, ferri-Tf complexes the transfused sTIR and thus reduces the effective free form of sTIR, which is an effect analogous to the transferrin injections in hypotransferrinemic animals.

Since the binding of sTIR to HFE has been already shown, the hypothesis proposed by Townsend and Drakesmith (28) and Cazzola et al. (53) may be probable, but other roles should also be taken into consideration, e.g. sTIR may influence the expression of the iron storage regulator hepcidin in the liver. It is supposed that the expression of this small peptide is regulated by HFE and a second transferrin receptor (TIR2) (24). In this case, the cross-talk between the cellular iron demand and the iron storage situation may be mediated by sTIR. The demonstration that sTIR also functions as a regulator of iron export from reticuloendothelial cells and duodenal enterocytes is currently under investigation.

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