How the binding of human transferrin primes the transferrin receptor potentiating iron release at endosomal pH

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Edited by Pamela J. Bjorkman, California Institute of Technology, Pasadena, CA, and approved June 23, 2011 (received for review April 12, 2011)

Delivery of iron to cells requires binding of two iron-containing human transferrin (hTF) molecules to the specific homodimeric transferrin receptor (TFR) on the cell surface. Through receptor-mediated endocytosis involving lower pH, salt, and an unidentified chelator, iron is rapidly released from hTF within the endosome. The crystal structure of a monomeric N-lobe hTF/TFR complex (3.22-Å resolution) features two binding motifs in the N lobe and one in the C lobe of hTF. Binding of Fe₃⁺hTF induces global and site-specific conformational changes within the TFR ectodomain. Specifically, movements at the TFR dimer interface appear to prime the TFR to undergo pH-induced movements that alter the hTF/TFR interaction. Iron release from each lobe then occurs by distinctly different mechanisms: Binding of His349 to the TFR (strengthened by protonation at low pH) controls iron release from the C lobe, whereas displacement of one N-lobe binding motif, in concert with the action of the dilysine trigger, elicits iron release from the N lobe. One binding motif in each lobe remains attached to the same α-helix in the TFR throughout the endocytic cycle. Collectively, the structure elucidates how the TFR accelerates iron release at endosomal pH.

Human serum transferrin (hTF) is an 80-kDa bilhaloglycoprotein synthesized by hepatocytes and secreted into the serum where it binds iron acquired from the diet (1). Ferric iron (Fe³⁺) is held extremely tightly within one or both of the homologous N and C lobes that comprise hTF for transport to cells throughout the body (2). Each lobe is composed of two subdomains (N1, N2 and C1, C2), forming a cleft within which the hexa-coordinate Fe³⁺ is bound to four amino acid ligands: one histidine, one aspartate, and two tyrosine residues. A synergistic anion, carbonate, anchored by a conserved arginine residue occupies the two remaining coordination sites in each cleft. Large-scale rigid body movements (approximately 50°) of the subdomains are observed when each cleft opens and iron is released (3,4).

Understanding the acquisition, distribution, and regulation of iron has been an active and important area of research for many years, as indicated by numerous excellent reviews (1,5–8). The redox properties of the Fe³⁺/Fe²⁺ pair are indispensable to the physiological functions of both electron and oxygen transport. In the absence of hTF, Fe³⁺ is highly insoluble and readily hydrolyzed; reduction to Fe²⁺ can produce reactive oxygen species via the Fenton reaction.

Cruceffe cient to Fe³⁺ delivery to cells is a TF specific homodimeric receptor, TFR, which binds two hTF molecules (9,10). A type II transmembrane glycoprotein, full-length TFR is comprised of a short N-terminal intracellular region (residues 1–67) containing an endocytosis motif (YXXφ), a transmembrane region (residues 68–88), and a stalk (residues 89–120) that connects to the large hTF binding ectodomain (121–760) (11). Although the stalk contains two disulfide bonds covalently linking the monomers, the TFR homodimer forms even in the absence of the stalk region. The 3.2-Å crystal structure of the TFR ectodomain revealed three distinct domains per monomer: the protease-like domain (domain I, 121–188 and 384–606), the apical domain (domain II, 189–383), and the helical domain (domain III, 607–760) (12).

In normal plasma, hTF is only approximately 30% iron saturated with a distribution of approximately 27% diteric hTF (Fe₂⁺hTF), 23% monoferric N (FeNhTF), 11% monoferric C (FeChTF), and 40% apohTF (13). Fe₂⁺hTF preferentially binds with nM affinity to the TFR at neutral pH, whereas hTF without iron (apohTF) binds poorly at this pH (6). The two monoferric species bind with a similar intermediate affinity demonstrating that each iron-containing lobe in the context of full-length hTF contributes equally to the binding isotherm (14,15). Thus it is possible to prepare stable complexes of either monoferric hTF species bound to the TFR that are physiologically relevant.

Iron within each lobe of hTF is transported into cells by receptor-mediated endocytosis in which lower pH approximately 5.6, the participation of the TFR, and an unidentified chelator within the endosome orchestrate the efficient and balanced release of iron from each lobe of hTF (16–19). Prior to exiting the endosome via the divalent metal transporter 1, Fe³⁺ must be reduced to Fe²⁺. The ferrireductase Steap3 may be involved in this process (20). Crucial to the recycling of hTF, after iron is released, apohTF remains bound to the TFR at the lower pH within the endosome and returns to the cell surface where it dissociates [or is displaced by Fe₂⁺hTF (21)].

Although the structure of apohTF has been determined (4), and despite tremendous effort, a crystal structure of Fe₂⁺hTF has been surprisingly elusive. Likewise, the hTF/TFR complex has evaded all efforts at crystallization, though the structures of TFR alone (12), TFR complexed with the HFE protein (22), and TFR complexed with a portion (GP1) of the Machupo virus (23) have been reported. In 2004, a 7.5-Å cryo-EM model of the hTF/TFR complex provided important insights into the molecular association of hTF and TFR (24). The validity of this model has been tested by mutagenesis studies; the contributions of specific residues in both hTF and the TFR to the binding isotherm have been measured by surface plasmon resonance (SPR) or isothermal titration calorimetry, as well as cell binding studies (25).

Here, we report the crystal structure of an hTF/TFR complex at a resolution of 3.22 Å. The improved resolution of the present structure reveals a number of unique features of the hTF/TFR interaction that have a direct impact on function and highlight the
unique receptor-mediated mechanisms of iron release from each lobe. Our structure provides previously undescribed targets for future studies to advance understanding of how the interaction between hTF and the TFR promotes iron release in a physiologically relevant time frame.

**Results**

**Overview of Structure.** The asymmetric unit contains two TFR sub-units (chains A and B) and two Fe₉₈hTF molecules (chains C and D), which represent two half-biological units. Each unit forms an independent biological assembly across a crystallographic twofold axis, such that chains A and C associate with a symmetry-related A′ and C′ (Fig. 1 and Fig. S1 symmetry molecules designated A or C′). Likewise, chain B and chain D form a separate biological assembly with a symmetry mate DB·B′ D′ (B′ or D′). The final model of Fe₉₈hTF in the complex is comprised of the N1, N2, and C1 subdomains. Unfortunately, insufficient electron density precluded placement of the C2 subdomain in the model. Each TFR monomer contained residues 121–758 with three N-linked glycans at Asn251,317, and 727, each fit with a single N-acetylglucosamine moiety.

Extensive contacts between the two TFR monomers form the noncovalent dimer burying significant surface area (approximately 3,200 Å²), with the helical domains from each monomer contributing substantially to this interaction. The surface area buried at the hTF/TFR interface (approximately 1,330 Å²) is less than half of the TFR dimer interface; the C lobe contributes approximately 60% and the N lobe contributes approximately 40% to this interface. Binding of Fe₉₈hTF to the TFR involves three primary interaction motifs. The regions of the N1 and N2 subdomains that contact the TFR (helical and protease-like domains, respectively) are located on either side of the hinge region of the N lobe. The C lobe of hTF only contacts the TFR (helical domain) through the C1 subdomain. Significantly, there is no experimental evidence to support the involvement of the C2 subdomain in binding to the TFR.

**hTF N1-TFR Motif.** The N1/TFR interface accounts for approximately 57% of the total contact surface area between the N lobe and TFR. The contacts of this motif are more extensive than suggested by the cryo-EM model. Nonadjacent residues Arg50 in helix α-2, Tyr68 and Tyr71 both in helix α-3, as well as Ala73 and Asn75 in a loop (residues 72–76) within the N1 subdomain of hTF are in contact with three residues in the helical domain of the TFR [Gly661 and Asn662 in αIII-3, and Glu664 in the αIII-3/αIII-4 loop (residues 663–667)] (Table S1 and Fig. 2A). Arg50 in hTF (not identified in the cryo-EM model) likely forms a salt bridge with Glu664. Tyr68 and Tyr71 from hTF hydrogen bond with Gly661 and Asn662 in the TFR. The backbone oxygen of Ala73 engages in a hydrogen bond with the backbone nitrogen of Glu664, whereas the nitrogen of Ala73 interacts with the backbone oxygen of Asn662 of the TFR. There is no clear pattern of conservation of the residues in this motif (4) that accounts for the specificity of the interaction between the N1 subdomain of hTF and the TFR. We suggest that the requirement for specific residues is somewhat obliterated by the presence of backbone interactions. This is consistent with the observation that mutation of either TFR residue Asn662 or Glu664 did not significantly affect binding of Fe₉₈hTF or apohTF to the TFR as measured by SPR (25).

**hTF N2-TFR Motif.** Two loops in the N2 subdomain of hTF interact with the N-terminal region of the TFR ectodomain and account for approximately 43% of the N lobe/TFR interface (Table S1 and Fig. 2B). Van der Waals and hydrophobic interactions occur between two proline residues (142 and 145) in the first loop of hTF (139–145) and Asp125 and Tyr123 in the TFR, respectively. Two residues in the second hTF loop (154–167), Asp166 and Phe167, also contact Tyr123 in the TFR. The side chain of Asp166 may hydrogens bond with both the backbone nitrogen of TFR Arg121 and the side chain OH of Tyr123 in the TFR. Asp166 and Phe167 were not predicted to interact with the TFR in the cryo-EM model. The importance of three of the four residues in the first loop (142–145, referred to as the PRKP loop) in the binding of the N2 subdomain has been unequivocally established (15) and is further

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**Fig. 1.** Structure of the Fe₉₈hTF/TFR complex. The biological TFR homodimer (TFR·TFR′, A·A′) with two Fe₉₈hTF (Fe₉₈hTF and Fe₉₈hTF, C·C′) molecules bound is shown oriented with the cell surface at the bottom. The TFR homodimer is colored according to the domains: The apical domain is blue, the protease-like domain is green, and the two monomers of the helical domain are brown and tan. The Ca₁⁺ bound within the apical domain of each TFR monomer is shown in yellow. The Fe₉₈hTF molecules are colored according to subdomain: N1 is gray, N2 is black, and C1 is purple. The bridge between the two lobes is cyan. The Fe⁺² bound within each N lobe of hTF is shown in red. All figures were prepared using Pymol (43).

**Fig. 2.** hTF N-lobe-TFR interaction motifs (also see Table S1). (A) Fe₉₈hTF residues involved in the N1 interaction motif (gray). Arg50, Tyr68, Tyr71, Ala73, and Asn75 within the N1 subdomain of hTF are close to three residues in the helical domain of the TFR (Gly661, Asn662, and Glu664). Residues Leu72 and Pro74, although involved in the N1 interaction motif, have been omitted for clarity. (B) Fe₉₈hTF residues involved in the N2 interaction motif (black). The space filling representation of the N2 motif emphasizes that the predominant mode of interaction is van der Waals with the N-terminal region of the TFR ectodomain and account for approximately 43% of the N lobe/TFR interface (Table S1 and Fig. 2B). Van der Waals and hydrophobic interactions occur between two proline residues (142 and 145) in the first loop of hTF (139–145) and Asp125 and Tyr123 in the TFR, respectively. Two residues in the second hTF loop (154–167), Asp166 and Phe167, also contact Tyr123 in the TFR. The side chain of Asp166 may hydrogen bond with both the backbone nitrogen of TFR Arg121 and the side chain OH of Tyr123 in the TFR. Asp166 and Phe167 were not predicted to interact with the TFR in the cryo-EM model. The importance of three of the four residues in the first loop (142–145, referred to as the PRKP loop) in the binding of the N2 subdomain has been unequivocally established (15) and is further
Conformational Changes in the TFR as a Result of hTF Binding. The binding of hTF results in the translation of the apical and protease-like domains of the TFR and the reorientation of the monoclonal antibodies within the homodimer. These changes are revealed by superimposing a TFR monomer from the HFE/TFR (PDB ID code 1DE4) and TFR alone (PDB ID code 1CX8) structures on our FeTf/hTF/TFR structure. The calculated changes in the mean rms show the effect of reorientation at the dimer interface in a ligand-dependent manner (Fig. 5, Inset). Site-specific changes per residue within a TFR subunit are highlighted by a positional comparison (Fig. 5). The TFR in the FeTf/hTF complex is very similar to the TFR in the HFE complex (black line), but varies considerably in comparison to the unliganded TFR (red line).

The most dramatic change in the TFR structure as a result of hTF binding is observed in the loop containing one of three glycosylation sites, Asn317 (Fig. 5, designated as TFR-TFR’ + C1 motif). Specifically, the helical domain and C terminus from one TFR monomer interacts with the loop containing the glycosylated Asn317 from the other TFR monomer, as well as with His318 (Table S2). Phe316 is shifted by 8 Å and His318 flips, bringing it to within 5 Å of the C terminus of the other TFR monomer (in comparison to a distance of 17.5 Å in unliganded TFR) (Fig. 4). Although the interaction between nearby Gin320 and Ser638 is unchanged, a number of rearrangements occur at the TFR dimer interface. Specifically, binding of hTF causes two Trp residues from the helical domain of one TFR monomer (Trp641 and 740) to undergo significant changes in packing with the other TFR monomer. Thus, Trp641 shifts from interacting with the side chain of Phe316 to interact with the backbones of Asn317 and His318. Trp740 changes from backbone packing and a carbonyl hydrogen bond with Pro314 to hydrophobic bonding with the backbone of Gly469 and F-stacking with Phe316, and His318 and Tyr470.

As in the HFE/TFR structure, binding of hTF causes a rotation along the TFR dimer interface bringing four histidines (His475 in the protease-like domain and His684 in the helical domain from each TFR monomer) into proximity (Fig. S3). The formation of this histidine cluster is another significant change in the TFR structure as a result of hTF binding (Fig. 5, designated TFR-TFR’ interface). Other changes are observed in the regions of the TFR involved in binding the various hTF motifs. Additionally, we suggest that conformational changes near residue Trp528 in
is atypical of occupancy by Ca

domain and the protease-like domain features a metal ion with

and C1 subdomains in our structure and the bridge in the Fe

vance, the position of the seven amino acid bridge between the N1

monomer. Asp376 (C1) observed in all three structures (FeNhTF from our

α

addition to multiple interactions of the N1 and C1 subdomains

appear to change as a function of iron status of the N lobe. In

N1 and C1 subdomains of hTF, relative to each other, does

map at 1 sigma (blue). (Inset) The crystal structure of TFR in complex with FeNhTF. The maps shown are for the anomalous difference Fourier for the data collected at 0.98 Å contoured at 3 sigma (red) and a simulated annealing composite omit map at 1 sigma (blue). (B) Overlay of A (darker shades) and the cryo-EM complex (1SUV) (24) after least-squares superposition using the TFR molecule (chain A). Secondary structural elements are labeled for clarity. Note that orientation has changed relative to Fig. 1, such that the cell surface is at the top. The Ca

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bound within the apical domain of each TFR monomer is shown in yellow.

the TFR are attributable to its proximity to the bridge between the N and C lobes of hTF.

Metal Binding Site in the TFR. The interface between the apical domain and the protease-like domain features a metal ion with octahedral coordination involving residues Thr310, Phe313, Glu465, and Gln468. Although significant anomalous scattering is atypical of occupancy by Ca

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or Mg

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(23). (Fig. S4), analysis of our recombinant TFR by inductively coupled plasma-mass spectrometry (ICP-MS) revealed the presence of a single Ca

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per monomer.

The N1 and C1 Motifs Remain Attached Throughout the Endocytic Cycle. The N1 and C1 subdomains are directly connected by a seven residue bridge (332–338) that joins the lobes of hTF. Least-squares superposition of the Fe

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rabbit TF structure onto the FeNhTF from our structure indicates that the position of the N1 and C1 subdomains of hTF, relative to each other, does not appear to change as a function of iron status of the C lobe (Fig. S5). Likewise, superposition of the apohTF structure onto the FeNhTF from our structure indicates that the position of the N1 and C1 subdomains of hTF, relative to each other, does not appear to change as a function of iron status of the N lobe. In addition to multiple interactions of the N1 and C1 subdomains with αIII-3 in the TFR, a salt bridge between Arg308 (N1) and Asp376 (C1) observed in all three structures (FeNhTF from our structure, Fe

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rabbit TF and apohTF) may help maintain their orientation relative to each other.

The Bridge Between the N and C Lobes. Of probable functional relevance, the position of the seven amino acid bridge between the N1 and C1 subdomains in our structure and the bridge in the Fe

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rabbit TF structure is very similar; in contrast, the bridge in the apohTF structure is in a very different orientation, implying that the loss of iron from the N lobe results in movement of the bridge residues.

Discussion

The crystal structure of the Fe

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hTF/TFR complex in combination with previous structures suggests a mechanistic basis for the kinetic differences in iron release from each lobe of hTF in the presence of the TFR. The structure of the complex clearly shows that the TFR is altered by hTF binding. The relative positions of the N1 and C1 subdomains of hTF appear to remain constant throughout the cycle, indicating that the N2 and C2 subdomains must move to accommodate the approximately 50° cleft opening and the release of iron from each lobe. Moreover, some insight into how apohTF is stabilized by and remains bound to the TFR throughout the endocytic cycle is provided.

Conformational Changes in TFR Induced by hTF Binding. A crucial finding is that Fe

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hTF binding at pH 7.4 repositions the TFR domains within each monomer, priming the TFR homodimer to undergo movements when the hTF/TFR complex encounters endosomal pH. Specifically, and as observed for the binding of HFE (22), binding of hTF to each TFR monomer causes a rotation at the dimer interface that brings four histidines (His475 in each protease-like domain and His684 in each helical domain) into proximity (Fig. S3). We suggest that binding of two hTF molecules is probably required to fully prime the TFR dimer. As previously suggested (22), endosomal pH triggers a chain reaction at the TFR dimer interface. Specifically, protonation of the histidine cluster would result in movement of the TFR, perturbing (but not severing) the interaction with bound hTF. This histidine cluster would be sensitive to pH changes that occur within the endosome; however, its location deep within the TFR dimer interface might restrict direct solvent access. We suggest that nearby solvent accessible Arg680 could serve as a proton shuttle from the endosomal milieu to the histidine cluster to circumvent this restriction. Because the N1 and C1 subdomains both remain bound to the same TFR helix (αIII-3), even a small pH-induced movement of the TFR could impact the stability of the iron binding cleft within each lobe. As detailed below, other elements in each lobe are simultaneously undergoing pH-induced changes that promote iron release. In contrast, protonation of both the TFR and HFE histidine residues completely disrupts the HFE/TFR interaction below pH 6.0 (22).

Fig. 4. Intersection formed between apical domain (blue) and protease-like domain (green) of one TFR monomer (TFR), the helical domain (brown-tan) of the other TFR monomer (TFR'), and the C1 subdomain (purple) of hTF. (A) Our crystal structure of TFR in complex with Fe

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hTF. The maps shown are for the anomalous difference Fourier for the data collected at 0.98 Å contoured at 3 sigma (red) and a simulated annealing composite omit map at 1 sigma (blue). (B) Overlay of A (darker shades) and the cryo-EM complex (1SUV) (24) after least-squares superposition using the TFR molecule (chain A). Secondary structural elements are labeled for clarity. Note that orientation has changed relative to Fig. 1, such that the cell surface is at the top. The Ca

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bound within the apical domain of each TFR monomer is shown in yellow.

Fig. 5. Plot of the root mean squared deviation calculated using CNS for chain A from the complex compared with a single chain from the receptor alone [red—1CX8 (12)] and when in complex with HFE [black—1DE4 (22)]. (Inset) The table shows the mean rms for both chains of the TFR dimer after superposition of the single chain, P, A, and H refer to the protease-like, apical, and helical domains of the TFR, respectively.

1CX8 − 1DE4

2

1DE4

P

A

H

1.24

2.03

2.18

3.44

www.pnas.org/cgi/doi/10.1073/pnas.1105786108

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Significantly, binding of hTF causes surface exposed His318 in the apical domain of each TFR subunit to flip into the intersection formed by the two TFR monomers and the C1 subdomain of hTF; His318 moves nearly 18 Å relative to the unliganded TFR structure (see below). Collectively, this region of the TFR influences both protein stability and iron release from hTF: The adverse effect of the N317D TFR mutant on expression level and iron release support its importance (29).

Kinetics of Iron Release from Each Lobe of hTF. Accurately describing the mechanism of iron release from each lobe has been challenging due to the number of variables (the TFR, endosomal pH, a chelator, salt, and cooperativity between lobes). We have recently reported a complete set of kinetic rate constants for conformational changes and iron release from hTF at pH 5.6 (± TFR) using an array of recombinant hTF constructs (19). Kinetic studies from Fe₂hTF at pH 5.6 (with EDTA as chelator) in the absence of the TFR indicate that 96% of the time iron is released quickly from the N lobe (17.7 min⁻¹), followed by slow release from the C lobe (0.65 min⁻¹) (Fig. S24) (19). Additionally, in the absence of the TFR there is clearly cooperativity between the two lobes of hTF. Specifically, iron release from the N lobe is sensitive to the C lobe (although the reverse is not true) (19, 30). Fitting of the kinetic data from the Fe₂hTF/TFR complex has allowed us to estimate that approximately 65% of the time iron is released first from the C lobe (kₑ₁C = 5.5 min⁻¹) and 35% of the time from the N lobe first (kₑ₁N = 2.8 min⁻¹) (19) (Fig. S2B). Together with previous TF structures, the present Fe₂hTF/TFR structure provides insights that help to explain the kinetic behavior of each lobe of hTF.

Iron Release from the C Lobe. Iron release from the C lobe of hTF in the absence of the TFR is extremely slow and unaffected by the N lobe (18). The C lobe features a triad of residues (Lys334-Arg632-Asp634) that appears to control the rate constant of iron release in the absence of the TFR (31, 32). Iron release from the C lobe in the presence of the TFR proceeds by a different mechanism and is 7- to 11-fold faster than in the absence of the TFR (19). Recent studies have demonstrated that iron release from the C lobe is dictated by His349, but only when hTF is bound to the TFR (28). Based on the cryo-EM structure, it was predicted that a hydrophobic patch (TFR residues Trp641 and Phe760) interacts with His349 and stimulates iron release by stabilizing the apoTF/TFR complex (27). Because of the 5-Å shift of helix α₁ of the C lobe in our structure, His349 actually lies in the intersection formed between the two TFR monomers and the C1 subdomain of hTF and is positioned to interact with several C-terminal residues (Asp757-Phe760) of the TFR (but not with Trp641).

The critical role of His349 as the driving force of TFR stimulated iron release from the C lobe is clearly demonstrated by the newly determined kinetics of iron removal from the H349A mutant in the Fe₂hTF/TFR complex whereby the rate constant for iron removal from the C lobe is reduced 12-fold (kₑ₂C = 7.2 versus 0.61 min⁻¹). In contrast to the Fe₂hTF/TFR control, which requires that both pathways be included in the fit, the data for the H349A mutant fit only to the single upper pathway (N lobe followed by the C lobe) (Fig. S2). Interestingly, the rate constant for iron release (kₑ₁C = 0.61 ± 0.02 min⁻¹) from the C lobe of the H349A Fe₂hTF/TFR complex is essentially identical to the rate constant of kₑ₂C = 0.65 ± 0.06 min⁻¹ for iron release from the C lobe of Fe₂hTF in the absence of the TFR (Fig. S24). We suggest that, in wild-type hTF, protonation of His349 at pH 5.6 converts a weak hydrophobic interaction with Phe760 at the C terminus of the TFR into either a stronger cation-π interaction with Phe760 or a salt bridge with Asp757, causing a conformational change in the C lobe and accelerating iron release from this lobe.

Iron Release from the N Lobe. In the absence of the TFR, iron release from the N lobe relies on protonation of a pair of lysines (Lys206 and Lys296 referred to as the dibysine trigger) on opposing sides of the binding cleft that form a hydrogen bond at neutral pH and literally trigger cleft opening at endosomal pH (33). The release of iron from the N lobe is further accelerated by binding of anions to Arg143, a recently identified kinetically significant anion binding (KISAB) site in the PRKP loop in the N2 subdomain (34). Attachment of both the N1 and N2 subdomains to the TFR limits access to this KISAB site, hinders cleft opening, and results in a rate of iron release that is 6- to 15-fold slower than in the absence of the TFR (19). Given that the N1 and C1 subdomains maintain their positions relative to each other in both the Fe₂ rabbit TF and apoTF structures, the N2 subdomain must engage from the TFR to allow the cleft to open. We suggest that a pH-induced movement of the TFR may help destabilize binding of the N2 subdomain, which is relatively weak. Rearrangement of the PRKP loop in the N lobe then pulls the N2 subdomain away from the TFR allowing the cleft to open and release of iron.

Stabilization of the apoTF/TFR Complex. The return of apoTF to the cell surface is a distinctive feature of the endocytic cycle. Therefore, release of iron from each lobe of the hTF/TFR complex must be effectively balanced with stabilization of the apo complex. The significant conformational changes associated with cleft opening and iron release from each lobe must be accommodated. The Fe₀hTF/TFR structure provides a molecular basis for the stabilization motifs within each lobe. Although some details of the interactions of the N1 and C1 subdomains with the TFR may change during the endocytic cycle, many of the interactions within these two binding motifs are probably preserved (Table S1); studies showing that mutation of TFR residues Asn629, Gly647, Phe650, or Arg651 affected binding of both Fe₂hTF and apoTF are consistent with this idea (25). In the C lobe, additional stabilization of the apo conformation is imparted by protonation of the two histidine residues (His549 and His581) at endosomal pH, which strengthens this region through potential cation-π interactions with Phe760 and Trp641 of the TFR, respectively. The unanticipated movement of His318 provides additional detail as to how the C1 subdomain remains bound to the TFR throughout the endocytic cycle. As revealed by the apoTF structure (4), the apo conformation of the N lobe is secured by a salt bridge between Asp240 in the N2 subdomain and Arg678 in the C1 subdomain. Additionally, the PRKP loop is connected to the bridge between the N1 and C1 subdomains by a disulfide bond between Cys137 in the N2 subdomain and Cys331 in the N1 subdomain. Significantly, in the apoTF structure, movement of the PRKP loop and the disulfide bond (Cys137-Cys331) repositions the bridge bringing it closer to the protease-like domain of the TFR to possibly further stabilize the apo conformation in a pH-dependent manner.

In conclusion, the crystal structure of the Fe₀hTF/TFR complex at neutral pH reveals a number of unique aspects of this dynamic system, allowing a more accurate description of the interactions that control iron release from each lobe in the presence of the TFR. Obviously, a single, static crystal structure cannot provide absolute temporal resolution, as multiple events are occurring nearly simultaneously as the pH surrounding the Fe₀hTF/TFR complex changes during endocytosis. Nevertheless, the functional significance of the induced conformational changes in the TFR structure is evidenced by its direct participation in promoting iron release from the C lobe and hindering it from the N lobe. The structure advances our understanding of the important interactions, the role of the TFR, and provides previously undescribed information to drive future work.

Materials and Methods

Production and Purification of Fe₀hTF and the Soluble Portion of the TFR. Recombinant nonglycosylated monoferic hTF, designated Fe₀hTF, contains mutations preventing iron acquisition by the C lobe (Y426F/Y517F) and glycosylation (N413D and N611D) and is produced in a BHK cell expression system (35). Likewise, the glycosylated ectodomain of the TFR (residues

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121–760) is produced and secreted into the tissue culture medium of transfected BHK cells (29). Both constructs contain a 6X-His tag at the N terminus, followed by a factor Xa cleavage site (IEGR), and are purified from the medium using nickel affinity chromatography followed by gel filtration. The homogeneity of each preparation is evaluated by SDS-PAGE. The histidine tags have not been removed from either construct.

The HTf/TFR complex is formed in the presence of excess Fe3+ for HTf and is latched by passage over a Sephacyl 300HR gel filtration column (15). Following concentration to 20 mg/mL in 100 mM NH4HCO3, crystals are grown at 20°C by the hanging drop vapor diffusion method. The protein solution is mixed at a 2 to 1 ratio with reservoir solution containing 100 mM Hepes pH 7.5, 4.6% PEG 3350, 200 mM MgCl2, and 5–20% 1,2-propanediol. Pale pink crystals with dimensions of 50 to approximately 500 μm develop in 1 to 10 d.

Data Processing, Structure Solution, and Refinement. Integration of diffraction images and data scaling were performed using HKL2000 (36). Molecular replacement solutions were found using Phaser (37) within CCP4 version 6.2 (38) and utilized TFR monomer (PDB id code 1CX8) (12), the HTf N-lobe (PDB id code 1A8E) (39), and C1/C2 subdomains of apoHTF (PDB id code 2HAU) (4) as search models (Table 53). A clear molecular replacement solution for two TFR monomers was found with P42,2 as the space group, each forming the biological TFR homodimer with a symmetric mate. As shown in Table 54, three independent models of Fe3+ HTf in the complex were generated using datasets derived from multiple crystallization and cryoprotection conditions and contain most of the N1, N2 and C1 subdomains. Preliminary solutions were improved by rigid body refinement with Refmac5.5 (40) using the HTf N-lobe and the C1/C2 subdomains of the ectodomain of the TFR. Structure refinement was performed with Crystallography and NMR System (CNS) (41) version 1.2 and model building using Coot (42). A single Fe3+ and the syngestic carbonate were clearly observed in each Fe3HTf (Fig. 54).

No density was observed for the 6X-His tag, the factor Xa cleavage site, or the first two to three residues of the N terminus. The final model of each TFR in the complex contained residues 121–758 with three N-linked glycans at Asn251, 317, and 727 each fitted with a single N-acetylgalactosamine moiety. Because the carbohydrate composition for BHK expressed TFR is unknown, the remaining density could not be unambiguously built. Again no density for the 6X-His tag and the factor Xa cleavage site at the N terminus of this construct was observed in the model of the TFR. The model derived from the 3.2 Å data was refined to an R factor of 27.2% (Rfree of 31.4%) with 99.5% in the preferred and allowed regions of the Ramachandran plot and 0.1% outliers (Table 54).

ACKNOWLEDGMENTS. We thank the University of Vermont Center for X-Ray Crystallography; Alexei Soares at the Brookhaven National Laboratory “Mail-“Translation Programs”. This study was measured at beamline X25 of the National Synchrotron Light Source with financial support from the Offices of Biological and Environmental Research and of Basic Energy Sciences of the US Department of Energy, and from the National Center for Research Resources (NCRR) of the National Institutes of Health (NIH) (P41RR012408). Chae Un Kim and Irina Kricunov at Macromolecular Diffraction at Cornell High Energy Synchrotron Source (CHESS) (MacCHESS) Beamline F2. Some of the work is based upon research conducted at the CHESS, which is supported by the National Science Foundation (NSF) and the NIH/NIAMS under P41-M003745 using MacCHESS facility, which is also supported by NIH Award RR-01646 through National Center for Research Resources. This work was funded by US Public Health Service R01 DK21739 (to A.B.M.) and R01 GM-20194 (to N.D.C.). Support for B.E.E. and A.N.S. came from Hemostasis and Thrombosis Training Grant ST32HL075794 issued to Delcy Rueff at the University of Vermont Moreron Blood Institute. A.N.S. is currently funded by an American Heart Association Predoctoral Fellowship (10PRE4200100).