Differential Effect of a His Tag at the N- and C-Termini: Functional Studies with Recombinant Human Serum Transferrin†

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ABSTRACT: Attachment of a cleavable hexa His tag is a common strategy for the production of recombinant proteins. Production of two recombinant nonglycosylated human serum transferrins (hTF-NG), containing a factor Xa cleavage site and a hexa His tag at the carboxyl terminus, has been described [Mason et al. (2001) *Prot. Exp. Purif 23*, 142−150]. More recently, hTF-NG with an amino-terminal His tag and a factor Xa cleavage site has been expressed (>30 mg/L) in baby hamster kidney cells and purified from the tissue culture medium. Although it is frequently assumed that addition of a His tag has little or no effect on function, this is not always confirmed experimentally. In the present study, in vitro quantitative data clearly shows that the presence of the C-terminal His tag has an effect on the release of iron from recombinant hTF at pH 7.4 and 5.6. Measurement of the rate of release from both the N- and C-lobes is reduced 2−4-fold. These findings provide further compelling evidence that the two lobes communicate with each other and highlight the importance of the C-terminal portion of the C-terminal lobe in this interaction. In contrast to these results, we demonstrate that the presence of a His tag at the N-terminus of hTF has no effect on the rate of iron release from either lobe. In a competition experiment, both unlabeled N- and C-terminal His-tagged constructs were equally effective at inhibiting the binding of radio-iodinated diferric glycosylated hTF from a commercial source to receptors on HeLa cells as the unlabeled recombinant diferric hTF-NG control. Thus, the presence of a His tag at either the N- or C-terminus of hTF-NG has no apparent effect on the ability of these hTF species to bind to transferrin receptors.

Transferrins are metal-binding glycoproteins that function in both the transport of iron to cells and as bacteriostatic agents in a variety of biological fluids (*1*, *2*). The present-day transferrins (including serum transferrins, lactoferrin, and ovotransferrin) have evolved by a series of gene duplication and fusion events (*3*); these modern transferrins consist of two homologous, globular lobes termed the N- and C-lobes. Each lobe is comprised of a deep cleft defined by two subdomains (termed N-I and N-II and C-I and C-II) containing a single metal ion-binding site. Structural data for many different transferrins show that each ferric ion is directly coordinated to the side chains of two tyrosine residues, one histidine residue, one aspartic acid residue, and two oxygens from the synergistic carbonate anion which is anchored by an arginine residue (*4*). A unique aspect of transferrin chemistry is that the iron-binding ligands are distant in the amino acid sequence occupying positions D63 (D392), Y95 (Y426), Y188 (Y517), and H249 (H585) for the N- and (C)-lobes of glycosylated human serum transferrin (hTF-Gly),[1] respectively (*5*). Also unique is the absolute requirement for the presence of a synergistic anion to obtain high-affinity iron binding. In the absence of a suitable synergistic anion, the iron is hydrolyzed because little or no binding occurs. Iron binding leads to a large conformational change in the hTF structure in which the subdomains in each lobe twist and come together to close the cleft over the metal (*4*, *6*).

Transferrin is present in human serum at a level of ∼3 mg/mL. This relatively high abundance makes the purification of the serum protein relatively facile. Nevertheless, production of human serum transferrin by recombinant expression allows for the mutation of individual amino acid residues in order to determine their specific contributions to

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[1] Abbreviations: TF, transferrin; hTF-Gly, commercially available glycosylated human serum transferrin; hTF-NG, recombinant nonglycosylated human serum transferrin; hTF-His-C1, recombinant human serum transferrin comprised of full length hTF with a Factor Xa cleavage site and a hexa His tag attached to the carboxy terminus of the protein; hTF-His-C2, recombinant human serum transferrin comprised of truncated hTF (missing the final three carboxy-terminal amino acid residues) with a factor Xa cleavage site and a hexa His tag attached to the carboxy terminus of the protein; N1-His-hTF, comprised of the signal peptide followed by amino acids 1−4 from hTF, a hexa His tag, a factor Xa site, and finally the coding region for amino acids 1−679 of hTF-NG; hTF/2N, the N-lobe of human serum transferrin; DMEM-F12, Dulbecco's modified Eagle's medium-Ham F-12 nutrient mixture; BHK cells, baby hamster kidney cells; MC, metal chelate; TMB, 3,3′,5,5′-tetramethylbenzidine; ESI, electrospray ionization; Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid; NTA, nitrilotriacetic acid; EDTA, ethylenediaminetetraacetate; oTF, ovotransferrin.

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function. An added advantage of recombinant expression is the ability to produce homogeneous proteins that can facilitate the determination of high-resolution crystal structures (*4*). In the case of hTF-Gly, the avoidance of blood-borne pathogens is also an important consideration. Serum hTF-Gly is often an essential component of tissue culture medium, especially when growing human cell lines. Bovine TF in fetal bovine serum binds very poorly to human TF receptors and therefore is not a good source of iron needed for cell growth and proliferation (*7*−*9*). For all of these reasons, there has been a concerted effort to develop and optimize expression systems for hTF (see below).

The presence of 38 cysteine residues in hTF, all of which are engaged in disulfide linkages (there are 8 in the N-lobe and 11 in the C-lobe), makes the expression of correctly folded protein a challenging problem. In addition, hTF has a leader sequence that must be removed after secretion; it also contains two carbohydrate attachment sites in the C-lobe. In contrast to the difficulties faced in producing recombinant transferrins, verifying that the proteins are functional once they are produced and isolated is relatively straightforward. For example, the ability to bind iron reversibly is easily determined by spectral analysis; iron binding leads to a visible absorbance maximum around 465 nm, resulting in a change in the protein solution from colorless to salmon pink.

The efforts to produce hTF in a variety of expression systems has been recently reviewed (*10*, *11*). In our baby hamster kidney (BHK) cell expression system, both glycosylated and nonglycosylated hTF have been expressed and shown by many criteria to be equivalent to plasma-derived hTF-Gly in both iron binding and interaction with cellular transferrin receptors (*12*). Interestingly, neither extensive nor varied glycosylation of the protein produced in the BHK system or the complete absence of glycosylation had an effect on the receptor binding affinity (*12*). Mutated hTFs have been used to investigate the role of specific residues in function (*13*, *14*). We have recently described the production and purification of two different C-terminal His-tagged hTFs (*10*). The aim of these studies was to prepare a recombinant hTF-NG that could be purified more easily and reproducibly since the recombinant protein is secreted into the tissue culture medium that contains a considerable amount of bovine TF. The separation problem is exacerbated in the case of mutated hTFs because their behavior on an anion exchange column is often changed by the introduction of the mutation. Particularly if the mutation results in reduced production of the protein, the ability to obtain purified recombinant hTF for functional and structural studies can be limiting (*13*).

In the present report, we describe production of an N-terminal His-tagged hTF and provide data from functional studies showing that, although the presence of a C-terminal His tag slows the rate of iron release from each lobe, the N-terminal His tag has no effect on the rate of release from either lobe. In addition, TF receptors present on HeLa cells recognize all of the His-tagged constructs equally well. These studies will allow a more efficient and convenient approach to production of mutations introduced into full-length hTF for use in functional studies.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium-Ham F-12 nutrient mixture (DMEM-F12), antibiotic-antimycotic solution (100X), and the Klenow fragment of DNA polymerase I were from Gibco-BRL-Life Technologies. Fetal bovine serum was obtained from Atlanta Biologicals (Norcross, GA) and was tested prior to use to ensure adequate growth of BHK cells. Ultroser G is a serum replacement from BioSepra, (Cergy, France). Bovine factor Xa was prepared in the laboratory of Dr. S. J. Everse. The Qiaquick Nucleotide Removal kit was from Qiagen, as was the Ni−NTA resin. Corning expanded surface roller bottles, and Dynatech Immunolon 4 Removawells were obtained from Fisher Scientific. The chromatographic resin, Poros 50 HQ and the metal chelate (MC), and QE columns were from PerSeptive Biosystems. A Hi-Prep 26/60 Sephacryl S-200HR column was from Amersham Pharmacia. Methotrexate from Cetus was purchased at a local hospital pharmacy and used for selection of plasmid containing cells. Centricon 30 microconcentrators, YM-30 ultrafiltration membranes, and a spiral cartridge concentrator (CH2PRS) fitted with an S1Y10 cartridge were from Millipore/Amicon. Human serum transferrin which is glycosylated was purchased from Boehringer Mannheim. Bovine serum albumin was from Sigma. Rabbit antimouse immunoglobulin G was from Southern Biological Associates. Immunopure NHS-LC-Biotin and Immunopure avidin-horseradish peroxidase were from Pierce. The TMB Microwell peroxidase substrate system was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). All other chemicals and reagents were of analytical grade. Milli-Q water was used to prepare all solutions.

A monoclonal antibody specific to the N-lobe of hTF was a generous gift from Dade Behring (Marburg, Germany). Biotinylated diferric hTF-Gly was prepared according to the instructions from Pierce at a level of approximately 2 biotin molecules per molecule of hTF. The biotinylated hTF was stored as a 50% glycerol solution at −20 °C.

Molecular Biology. As described previously, two different C-terminal His-tagged hTF constructs have been prepared (*10*). One, designated hTF-His-C1, comprised the full-length nonglycosylated hTF (residues 1−679) to which a factor Xa site and a hexa His tag were added to the carboxy-terminal end. The nonglycosylated hTF was produced by mutation of both the Asn linkage sites at positions 413 and 611 to Asp residues (*12*). The second construct, designated hTF-His-C2, was identical except that it contained a truncated hTF-NG (residues 1−676). Details regarding the oligonucleotides for the C-terminal His-tagged constructs and insertion of the mutated hTFs into the pNUT vector are described elsewhere (*10*). Both constructs contained the hTF signal peptide to promote secretion from the BHK cells.

A third construct, designated N1-His-hTF, was comprised of the signal peptide followed by amino acids 1−4 from hTF, a His tag, a factor Xa site, and finally the coding region for amino acids 1−679 of hTF-NG. The hexa histidine tag and factor Xa cleavage site were incorporated into the N-terminal region of full-length hTF-NG by using in vitro mutagenesis in a three step procedure as follows:

Step 1: A double-stranded oligonucleotide containing a SmaI site (bold/italics), the upstream region of the signal peptide, and an AvrII site (bold) was constructed by hybridizing together the two synthetic oligonucleotides 5′-AAA***CCCGGG***AAGATGAGGCTCGCCGTGGGAGCCCTGCTGGTC-3′ and 5′-TAT**CCTAGG**ACGGCGCAGA-

CCAGCAGGGCTCCCACGGCGAGC-3′.

The oligonucleotides (2.0 μg/μL each) were heated at 85 °C for 30 min, allowed to cool slowly to room temperature, and then placed at 4 °C overnight. The ends of the resulting double-stranded DNA fragment were made blunt with the Klenow fragment of DNA polymerase I in the presence of 10mM dNTPs. The blunt-ended DNA fragment was purified using the Qiaquick Nucleotide Removal Kit, digested with SmaI and AvrII, and the new fragment was isolated by gel electrophoresis and Qiaquick gel extraction.

Step 2: A hexa His-tagged oligonucleotide was introduced into the full-length hTF-NG using a PCR-based procedure (*15*). In vitro mutagenesis was performed with the mutagenic oligonucleotide (forward)

5′ ATA***CCTAGG***GCTGTGTCTGGCTGTCCCTGATAAA-*CATCATCATCATCATCAT***ATCGAGGGAAGG***GTCCCT-GATAAAACTGTG-3′ (the AvrII site is italicized and bolded, the hexa-His tag is italicized, and codons for the factor Xa site are bolded) and an internal hTF primer (reverse) 5′-GCAACAGCATAATAGAAAGTC-3′ with Bluescript hTF/2N as the template. The conditions for the PCR reactions were 30 cycles with denaturation at 94 °C for 30 s, annealing at 48° for 30 s and extension at 72 °C for 30 s, and a final extension time of 10 min at 72 °C. The resulting DNA fragment containing the His tag was digested with AvrII and BamHI and the fragment was isolated as described above.

Step 3: The plasmid hTF-NG was digested with BamHI and XhoI and the resulting cDNA fragment isolated as before. The final construct was then formed by ligating together the three DNA fragments: (1) the signal peptide fragment with SmaI and AvrII ends, (2) the His tag and factor Xa site fragment with AvrII and BamHI ends, and (3) the remainder of the hTF-NG cDNA sequence with BamHI and XhoI ends. The completed insert was cloned into the SmaI and XhoI sites of Bluescript, and the mixture was used to transform *Escherichia coli* strain DH5α. Restriction mapping and DNA sequence analysis showed that one clone contained the correct orientation of fragments. The transferrin cDNA was then ligated into the SmaI site of pNUT (*16*) to give pNUT N His6 HTF NG. The correct orientation of the transferrin cDNA sequence in pNUT was determined by DNA sequence analysis prior to introduction into BHK cells.

The pNUT expression vector features a mouse metallothionein promoter to regulate expression of a cloned gene and a mutated dihydrofolate reductase that allows for rapid selection of cells containing multiple copies of the plasmid by using a high concentration of methotrexate. BHK cells were transfected and selected with methotrexate as previously described (*17*; *18*). Following the selection process, which takes between 10 and 20 days, the transformed cells containing the pNUT-hTF plasmids were expanded in flasks for transfer into roller bottles or frozen in 95% FBS/5% DMSO in a liquid nitrogen apparatus. The freezing, passage, and expansion of cells have been described in detail previously (*12*).

Production and Purification of the Recombinant Proteins. The details of the production and purification of the recombinant hTFs with and without C-terminal His tags have been reported elsewhere (*10*). Initially, purification of N1-His-hTF followed the same protocol, namely, concentration and exchange on a spiral concentrator, followed by chromatography on a Poros 50 HQ column to remove phenol red and most of the serum albumin present in the media. The final step of purification involved use of a Poros MC/M column (4.6 mm × 100 mm) loaded with copper and run using a BioCad Sprint chromatography system. The capacity of the column was 3−5 mg of His-tagged protein, so multiple runs were required. In purification of later batches of N1−His-hTF, a Qiagen Ni−NTA column was used to isolate the His-tagged hTF based on a protocol describing the purification of the soluble TF receptor (*19*). In these preparations the sample from the Poros 50 HQ column was exchanged on an Amicon stirred cell into start buffer (50mM Tris, pH 7.5, containing 300 mM NaCl, 20 mM imidazole, 10% glycerol, and 0.05% NaN_3), filtered through a 0.2 μm Acrocap filter, and pumped at a rate of 1 mL/min onto a column containing 5−10 mL of Ni−NTA resin (binding capacity 5−10 mg/mL). The column was run on the Sprint system allowing for continuous monitoring of the absorbance at 280 nm, the conductivity, and the pH. The column was washed with the start buffer until the baseline returned to the background level, and the His-tagged hTF was eluted with start buffer containing 250 mM imidazole. Fractions of 2 mL were collected. The eluted sample was pooled, concentrated, and passed over a Sephacryl S200HR column in 0.1 M ammonium bicarbonate to eliminate the imidazole, glycerol, and other buffer components. As described previously, the recombinant transferrins are maintained in an iron saturated form throughout the purification procedure by addition of iron NTA to each batch of the tissue culture supernatant (10).

Assay for Recombinant hTF Production. The amount of recombinant hTF-NG in the tissue culture medium and at various stages of the purification was determined by adaptation of a competitive solid-phase radioimmunoassay in which biotinylated hTF-Gly was substituted for iodinated sample (*10*).

Kinetics of Iron Release at pH 7.4. Iron removal from the transferrin samples was measured by monitoring the absorbance change at 480 nm for release of iron to the chelator Tiron using a Cary 50 Bio spectrophotometer (Varian, Inc). A circulating water bath maintained the temperature at 25 °C during the experiment, and the times were adjusted to ensure that iron removal from the proteins was complete (3−4 half-lives). Approximately 6 μM of protein and 12 mM of Tiron in a total volume of 250 μL of 100 mM HEPES buffer, pH 7.4, was used for the kinetic assays. The curve resulting from a plot of absorbance versus time was fitted using Origin software (Microcal, Inc). A single-exponential function and double-exponential functions were used to fit the recombinant N-lobe and full-length hTFs, respectively. For the diferric transferrin samples, the amplitudes of absorbance for two rate constants were averaged and fixed with the assumption that complete iron removal from each binding site contributes equally to the absorbance of the iron−Tiron complex which is being monitored. For all data reported, highly correlated fits ($R^2 > 0.99$) were obtained.

Kinetics of Iron Release at pH 5.6. In this case, iron removal from the transferrin samples was measured by monitoring the absorbance change at 470 nm for release of iron from the protein to the chelator EDTA using a Cary 50 Bio spectrophotometer as above (25 °C, complete iron removal). Approximately 6 μM of protein and 4 mM EDTA

in a total volume of 500 μL of 100 mM MES buffer, pH 5.6, was used for the assays. A double exponential function was used to fit the data obtained for the hTF-His-C1 and -C2 samples as described above. For hTF-Gly, hTF-NG, and N1-His-hTF, the rate of release from the N-lobe was too rapid to accurately measure on the Cary 50 under the conditions used. Instead, the first two minutes of collected data (attributed to the N-lobe release) were omitted from the analysis, and the remaining data were fit using a single-exponential function ($R^2 > 0.99$); the measured rate constant is attributed to the slower releasing C-lobe.

Mass Spectrometry. Electrospray (ESI) mass spectra of all protein samples were obtained using a JMS-700 MStation (JEOL, Tokyo, Japan) two-sector mass spectrometer equipped with a standard ESI source. Solutions of protein in 100 mM ammonium bicarbonate were diluted in $H_2O/CH_3OH/CH_3CO_2H$ (47:50:3, v:v:v) to a final concentration of approximately 5 μM and were continuously injected into the source at a rate of 5 μL/min. The spray needle potential was kept below 1.9 kV to avoid in-source oxidation of the protein ions. Acceleration voltage was kept at 5 kV, and the resolution was set at 1000. All spectra were recorded by scanning the magnet at a rate of 5 s/decade. Prior to data acquisition, the instrument was calibrated in the desired mass range using either cesium iodide in fast atom bombardment mode or a solution of apo-hTF/2N in $H_2O/CH_3OH/CH_3CO_2H$ (47:50:3, v:v:v) in ESI mode. Typically, each spectrum was an average of 80−180 scans.

Cleavage with Factor X_a. To cleave the His tag at the factor Xa concensus sequence, Ile-Glu-Gly-Arg, we exchanged the apo His-tagged proteins into 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 5 mM $CaCl_2$. A 1:100 (w:w) ratio of bovine factor Xa to recombinant protein was used to initiate the digestion which was incubated for 2 h at 25 °C with gentle rocking. An additional aliquot of bovine Xa equal to the original amount was added, and incubation continued for 3 h. Each sample was reloaded with iron-NTA and exchanged into the start buffer for application to the MC column. The cleaved portion not retained by the column was applied to a Poros QE/M anion exchange column for final purification and exchanged into HEPES buffer. A portion of the sample was removed and exchanged into 100 mM ammonium bicarbonate for analysis by mass spectrometry.

Cell Binding Experiments. In general, the protocols for preparing the HeLa S_3 cells, conducting the binding experiments and analyzing the data have been reported previously and were followed with the minor changes as noted below (*7*, *12*). In brief, iron-saturated hTF-Gly was iodinated with ^{125}I to a specific activity of 125 500 cpm/pmol using the McFarlane procedure (*20*). HeLa cells from three T-150 flasks were harvested using Versene and resuspended in 20 mM HEPES, pH 7.4, containing 10 mM $NaHCO_3$, 150 mM NaCl, and 2% bovine serum albumin. The cells were incubated for 20 min in buffer that also contained 20 mM NH_4Cl to inhibit iron removal from the diferric hTF samples in the subsequent incubations. Suspended cells (200 μL containing $\sim 3.5 \times 10^6$ cells) were added to separate 12 mm $\times$ 75 mm plastic tubes each containing Fe_2 ^{125}I -hTF (6.4 pmol) and six different amounts (ranging from 4 to 128 pmol) of unlabeled sample in a 100 μL. After 30 min of incubation at 37 °C with gentle agitation, portions of the cell suspension (100 μL $\times$ 2) were washed and assayed as described previously (*12*). The data were plotted as a log/logit plot. The log of the pmole/mL at logit $B/B_0 = 0$ was used to determine the amount of unlabeled protein required to inhibit 50% of the radioiodinated hTF binding to the HeLa cell hTF receptors.

Table 1: Summary of Spectral Characteristics for Iron-Loaded hTF and His-Tagged hTF

Fe_2hTF−CO_3 complexes				
protein	λ_{max} (nm)	λ_{min} (nm)	A_{max}/A_{min}	A_{280}/A_{max}
Fe_2hTF−Gly	457	414	1.12	25.5
Fe_2hTF−NG[a]	464	403	1.38	27.5
Fe_2hTF−His-C1[a]	461	397	1.19	24.6
Fe_2hTF−His-C2[a]	462	397	1.14	25.7
Fe_2N1−His-hTF	466	403	1.13	21.3

[a] Data from ref *10*.

RESULTS AND DISCUSSION

Expression of His-Tagged Proteins. The production and purification of the N-terminal His-tagged hTF, (N1−His-hTF) was comparable to the previous results obtained for the two C-lobe His-tagged constructs with a level of production of 34.1 ± 5.2 mg/L (*10*). Although the maximum production of all of the secreted recombinant His-tagged hTF is somewhat lower, on average, than that found for hTF-NG, the ease of the purification procedure compensates for this discrepancy. Following purification, we recovered 50−75% of the expressed N1-His-hTF regardless of which resin was used (the Poros MC containing copper or the Qiagen Ni−NTA). Chromatography on the Qiagen column is more convenient because the column size is scalable, thereby requiring fewer runs. In addition, serum albumin, which contains a copper-binding site and binds to the copper loaded Poros MC column, does not bind to the Qiagen Ni−NTA column. This eliminates the need to completely exclude all of the albumin prior to this step in the purification. Bovine serum albumin is the major protein in the serum replacement Ultroser G. Efforts to load the MC column with nickel instead of with copper resulted in a reduced binding capacity.

Characterization. Following purification, the hTF-His-C1, hTF-His-C2, and N1-His-hTF were compared to recombinant hTF-NG and were evaluated by a number of different criteria. The spectral properties of the diferric form of the various His-tagged hTFs are presented in Table 1 and compared to both hTF and hTF-NG. The spectral data indicates minimal disruption of the iron-binding sphere for each of the constructs implying that both lobes of the recombinant proteins are properly folded. Given that the iron-binding residues are far apart in the primary sequence (see Introduction), iron binding will not take place in the absence of correct folding and the proper formation of the 19 disulfide bonds. Since reversible iron binding is the benchmark of functional hTF, the spectral data are critical in validating that the various recombinant hTFs have spectral properties consistent with the ability to bind iron in both lobes and complete saturation of both lobes.

Analysis by ESI-MS was used to confirm the identity and integrity of the His-tagged hTF constructs. The ESI-MS results are presented in Table 2 and show that the signal peptide was cleaved during the expression and secretion

Table 2: Electrospray Mass Analysis of Recombinant hTF ± a Hexa His Tag

protein	calculated m_r	experimental m_r
hTF-NG[a]	75 143	75 142 ± 3.0
hTF-His-C1[a]	76 537	76 536 ± 3.8
hTF-His-C1-cleaved	75 601	75 613 ± 15
hTF-His-C2[a]	76 128	76 125 ± 7.2
hTF-His-C2-cleaved	75 192	75 204 ± 15
N1-His-hTF	76 861	76 883 ± 15
N1-His-hTF-cleaved	75 143	75 155 ± 20

[a] Data from ref *10*.

Table 3: Rate Constants for Iron Release from Transferrin by Tiron at pH 7.4, 25 °C

protein	k_n (min^{-1} × 10^3)	k_c (min^{-1} × 10^3)	number (n)	time (min)	ratio (k_n/k_c)
hTF-Gly	32.3 ± 1.4	8.5 ± 0.6	4	240	3.8
hTF-NG	25.9 ± 1.3	6.4 ± 0.5	8	240	4.0
hTF-His-C1	8.9 ± 0.3	3.6 ± 0.3	5	600	2.4
hTF-His-C1-cleaved	10.5 ± 1.0	4.8 ± 1.2	4	600	2.2
hTF-His-C2	8.4 ± 0.3	2.3 ± 0.1	4	720	3.6
hTF-His-C2-cleaved	12.4 ± 0.4	4.6 ± 0.4	3	600	2.7
N1-His-hTF	22.7 ± 0.9	6.4 ± 0.6	4	400	3.5
N1-His-hTF-cleaved	23.8 ± 1.2	6.3 ± 0.5	4	400	3.8
N-Lobe of hTF	26.7 ± 0.6	none	6	100	−

process and that the masses are completely consistent with the cDNA used to code for each construct. The ESI-MS results also show that factor Xa cleavage was successful in removing the hexa His-tag. The C-terminal His-tagged constructs should contain either full-length hTF (residues 1−679) in hTF-His-C1 or truncated hTF (1−676) in hTF-His-C2, with an addition of four extra amino acids (Ile-Glu-Gly-Arg) derived from the cleavage site. Alternatively, cleavage of the N-terminal His-tagged construct should yield only the parent protein (residues 1−679) since the Arg residue which is the target of the factor Xa immediately precedes the Val at position 1 of the mature hTF. Although the Arg is usually followed by an Ile residue in the concensus sequence for factor Xa, the substitution of the Val does not appear to have any adverse effect on the removal of the hexa His tag. The masses of the cleaved samples given in Table 2 indicate that the predicted results were obtained. Under the conditions used, the cleavage efficiency was about 80% for all three constructs, although the recovery of cleaved product was only about 50% of theoretical (data not shown) in each case. It is possible that either longer digestion times and/or additional enzyme might result in complete cleavage, although our functional data may preclude further efforts in this regard (see below).

Kinetics of Iron Release at pH 7.4. To assay function, the rate constants were determined for the release of iron from each site in hTF-Gly, hTF-NG, and the three His-tagged constructs. The results of these experiments are presented in Table 3. Immediately obvious is a 2−4-fold observed difference in the iron release rate between the N-lobe and the C-lobe for all the recombinant hTFs and for the control, hTF-Gly. Thus at pH 7.4, the iron release rate from the N-lobe is faster than the rate of release from the C-lobe for each protein. This finding is consistent with previous results in which the release of iron from plasma-derived hTF-Gly was measured (*21*−*23*) (although the ratio found varies with the conditions and with the particular chelator used). Of major significance is the observation that the addition of the hexa His tag to the N- or C-lobe has differential effects on the rate of iron release. Thus, the release of iron from both lobes in each of the C-terminal His-tagged hTF samples is 2−4 times slower than the rates measured in the non-His-tagged constructs and the N-terminal His-tagged construct. Obviously the presence of the factor Xa cleavage site and the hexa His tag significantly influence not only the C-lobe in which it resides but also the N-lobe. The effect is especially apparent in the mutant which lacks the final three amino acids of the hTF sequence (hTF-His-C2), in which the rate of iron release from the C-lobe compared to the wild type C-lobe is 2.8-fold slower under identical conditions. Removal of the His tag from the C1 construct results in a preparation with rates that are not significantly different from the uncleaved protein. Removal of the His tag from the C2 construct leads to somewhat faster rates of release from each lobe but does not yield rates equivalent to those found for the non-His-tagged hTF-NG. These findings imply that even the presence of four extra amino acids at the carboxy-terminus has an effect on the rate of iron release.

Table 4: Rate Constants for Iron Release from Transferrin by EDTA at pH 5.6, 25 °C

protein	k_n (min^{-1} × 10^3)	k_c (min^{-1} × 10^3)	number (n)	time (min)	ratio (k_n/k_c)
hTF-Gly	too fast	24.6 ± 0.8	3	200	−
hTF-NG	too fast	21.2 ± 0.6	4	200	−
hTF-His-C1	65.1 ± 2.6	14.0 ± 0.6	3	200	4.7
hTF-His-C2	34.4 ± 1.3	6.6 ± 0.3	3	230	5.2
N1-His-hTF	too fast	21.0 ± 0.6	3	160	−
hTF/2N	too fast	none			

The data presented in Table 3 supports the idea that the N- and C-lobes interact with each other and highlight the fact that relatively small changes can result in functional differences. Work by Williams and Moreton (*24*) with truncated half-molecules of ovotransferrin (oTF) and from our own studies (*25*) with recombinant oTF and oTF N- and C-lobes clearly showed that the C-terminal portion of each lobe is critical for the noncovalent interaction of the lobes in solution. Titration calorimetry provided a quantitative estimate of this interaction for the isolated oTF lobes (*25*), as well as for the recombinant N- and C-lobes of hTF (*26*). The X-ray structure of diferric oTF allowed for the identification of the residues making up hydrophobic patches that reside in the C-terminal portion of each lobe and accounts for at least some of this interaction (*27*). In the unpublished structure of monoferric hTF, again the C-terminus of the C-lobe resides in the region where the two lobes come together (*28*). The recent publication of the higher resolution pig and rabbit TF structures agrees with this observation (*29*).

Kinetics of Iron Release at pH 5.6. The results of iron release studies done at the putative pH of the endosome, pH 5.6, are presented in Table 4. It is important to emphasize that these release rates cannot be directly compared to those determined at pH 7.4 (Table 3) because both the buffers and chelators are different. Nevertheless, it is obvious that the data qualitatively agrees with the pH 7.4 results. The differences between both the hTF-His-C1 and -C2 constructs compared to each other and to the hTF-Gly, the hTF-NG, or the N1−His-hTF constructs are accentuated at this lower pH. As seen in Table 4, the rate of iron release from the

Table 5: Competition Assay: Inhibition of Binding of Radioiodinated Diferric hTF to Hela S_3 Cell Receptors by Recombinant Diferric hTF with and without a Hexa His Tag

protein	I_t[a] (nm)	average I_t (nm)	r^{2b}
Fe_2-hTF Gly	59.3	59.3 ± 4.9	0.995
(control)	65.3		0.990
	53.4		0.995
Fe_2-hTF-NG	22.0	21.5 ± 0.6	0.999
	20.9		0.991
Fe_2-hTF-His-C1	25.6	23.4 ± 2.2	0.998
	21.2		0.993
Fe_2-hTF-His-C2	21.0	21.2 ± 0.2	0.998
	21.4		0.986
Fe_2-N1-His-hTF	19.7	19.5 ± 0.7	0.990
	18.6		0.997
	20.2		0.995

[a] I_t is the total inhibitor concentration at 50% inhibition of radioiodinated Fe_2-hTF-Gly. [b] R^2 is the correlation coefficient for the straight line derived from plotting the log of the pmole/mL against logit B/B_0. As described in Methods, I_t is found at logit $B/B_0 = 0$.

N-lobe was too fast to accurately measure under the conditions used for the hTF-Gly, the hTF-NG, and the N1−His-hTF constructs, but was determined to be approximately twice as fast for the hTF-His-C1 versus the hTF-His-C2 construct. Likewise, the release rates for the C-lobes of these two constructs differed by a factor of 2, resulting in a similar k_N/k_C ratio of about 5.0.

N-Terminal His-Tagged hTF. In contrast to the C-terminal His-tagged hTF constructs, the N-terminal His-tagged hTF shows no significant difference in the rate of release from either lobe compared to hTF-NG, and the His tag has no significant effect on either rate at pH 7.4 or 5.6. This finding indicates that the N-terminal His-tagged hTF-NG is a good candidate for introduction of mutations into either lobe (Tables 3 and 4). Initially we plan to use this construct to evaluate mutations in the C-lobe since our efforts to produce recombinant C-lobe have not led to satisfactory levels of expression (*31*). Zak and Aisen (*32*) recently reported an alternative strategy in which full-length hTF is produced with a factor Xa cleavage site located in the hinge region between the two lobes. Cleavage with factor Xa leads to production of C-lobe that is able to bind to receptor with a K_d that is approximately 60-fold weaker than that observed for diferric hTF. This isolated C-lobe will be very useful, although the initial yield of recombinant protein appears to be lower than that of our His-tagged constructs. We think that evaluation of the hTF interaction with the TF receptor will be simpler using full-length hTF constructs since there is general agreement that the N-lobe plays a role in receptor binding, although the exact nature of this role is not completely clear.

Cell Binding. Experiments were performed to determine the ability of the different His-tagged hTF-NG constructs to compete with radioiodinated, diferric hTF for binding to TF receptors on HeLa S_3 cells. The simplest protocol for conducting such experiments is to add a constant amount of radiodinated hTF to a constant number of cells in the presence of increasing concentrations of unlabeled samples. The concentration of unlabeled sample required to inhibit 50% of the binding is reported for each of the recombinant proteins and compared to the Boehringer Mannheim hTF-Gly standard (Table 5). It is clear from these experiments that the TF receptor does not discriminate against any of the recombinant hTFs whether a His tag is present or not. In fact, all of the recombinant hTFs compete more effectively (by a factor of 3) with the iodinated diferric hTF-Gly compared to the unlabeled diferric hTF-Gly. Our previous studies using recombinant hTF-NG had led us to the conclusion that the carbohydrate played no role in receptor interaction (*12*). The current findings imply that the glycan may possibly have a negative impact on the interaction. We attribute the difference between the two studies to improvement in our ability to purify and quantitate the recombinant nonglycosylated hTF and/or to possible differences in the hTF purchased from Boehringer Mannheim.

The use of a His tag in expression of recombinant proteins from many different systems is becoming increasingly common. There is substantial literature documenting the effect (*33*, *34*) or lack of an effect when a His tag is added to a protein (*35*−*39*). Unfortunately, evaluation of this literature is often difficult because the "activity" of the His-tagged protein is not directly compared to a non-His-tagged control (*40*−*42*). Most frequently the His tag is located at the amino terminus of a recombinant protein produced in bacteria and is not secreted. In a large number of cases, the His tag is removed, but no report of the cleaved protein compared to the uncleaved protein is provided.

In conclusion, we have produced and purified substantial amounts of recombinant hTF with a hexa histidine tag and factor Xa cleavage site either at the N- or the C-terminus. We have verified the identity of the constructs and presented in vitro data showing that the presence of the tag at the C-terminus slows the rate of iron release from each lobe at both pH 7.4 and 5.6. The presence of the His tag at the N-terminus has no effect on the rate of iron release from either lobe compared to hTF-NG. Interestingly, all of the His-tagged constructs bind equally well to the TF receptor on HeLa S_3 cells. Future work will use the N-terminal His-tagged construct to query the role of specific residues in the C-lobe using mutagenesis.

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