The Cytoplasmic Domain of Transferrin Receptor 2 Dictates Its Stability and Response to Holo-transferrin in Hep3B Cells

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Transferrin receptor 2 (TfR2) is a homolog of transferrin receptor 1 (TfR1). TfR1 is ubiquitously expressed in most transferrin types with the notable exception of mature erythroid cells, whereas TfR2 is predominantly expressed in the liver (1–3). Like TfR1, TfR2 binds transferrin (Tf) in a pH-dependent manner but with 25-fold lower affinity and delivers iron to cells (4, 5). The regulation of TfR1 and TfR2 is distinctly different. TfR1 is inversely regulated at the mRNA level by an intracellular iron pool through interaction of the cytosolic iron regulatory protein with iron-responsive elements in the 3′-untranslated region of TfR1 mRNA (6). By contrast, TfR2 mRNA is not regulated by this mechanism. In keeping with this finding, no iron-responsive element is discernable in TfR2 mRNA (2, 3, 5, 7).

TfR2 is regulated at the level of protein stability by a novel mechanism. Unlike most receptors identified to date, Tf binding to TfR2 stabilizes TfR2 (8, 9). Most receptors either cycle independently of their ligand and their half-life is not affected by ligand binding as is the case for TfR1 (10) or upon ligand binding are targeted for degradation in the lysosome, like the epidermal growth factor (11). In contrast, the half-life of TfR2 increases in response to holo-Tf treatment over a physiological range of holo-Tf concentrations (8, 9), indicating that TfR2 correlates with changes in Tf saturation. The response of TfR2 to holo-Tf appears to be hepatocyte-specific. Non-hepatic cell lines that either endogenously express TfR2 such as K562 cells or are transfected with a plasmid coding for TfR2 do not show a similar response (7–9, 12).

Understanding which domain is important for the stability and trafficking of TfR2 is crucial for understanding its function in iron homeostasis. Although the ectodomains of TfR1 and TfR2 show 45% identity and 66% similarity, their cytoplasmic domains bear no significant similarity. In this study we constructed two chimeras consisting of different domains of TfR1 and TfR2 and investigated their response to holo-Tf to map which domain of TfR2 is responsible for its stabilization in a hepatoma cell line that does not express detectable levels of TfR2. We found that although Tf binding is necessary for the increase in stability, it did not matter which Tf binding domain was utilized. The cytoplasmic domain of TfR2 is largely responsible for its stabilization by holo-Tf.

MATERIALS AND METHODS

Chimera Plasmid Construction—The templates for PCR amplification of TfR1 and TfR2 are fTR/pUHD10-3 (13) and pCDNA3.1-TfR2, respectively. For the chimera of TfR2-cyto/TfR1-tm-ecto-FLAG, a fragment of TfR2 cytoplasmic domain (amino acids (aa) 1–80) was amplified using primers 5′-CTG-GATCCATGGAGCGGCTTTGGGTCAT-3′ (forward) and 5′-CAGATACTTCCACTACACCTCCGTCCTGCTGCC-3′ (reverse); a fragment of TfR1 transmembrane and ectodomain (aa 75–80 and TfR1 aa 62–63, reverse), a fragment with iron-loaded transferrin (holo-Tf) into cells. Unlike the ubiquitously expressed TfR1, TfR2 is predominantly expressed in the liver. Mutations in TfR2 gene cause a rare autosomal recessive form of the iron overload disease, hereditary hemochromatosis. Previous studies demonstrated that holo-Tf increases TfR2 levels by stabilizing TfR2 at the protein level. In this study we constructed two chimeras, one of which had the cytoplasmic domain of TfR2 and the remaining portion of TfR1 and the other with the cytoplasmic and transmembrane domain of TfR1 joined to the ectodomain of TfR2. Similar to TfR2, the levels of the chimera containing only the cytoplasmic domain of TfR2 increased in a time- and dose-dependent manner after the addition of holo-Tf to the medium. The half-life of the chimera increased 2.7-fold in cells exposed to holo-Tf like the endogenous TfR2 in HepG2 cells. Like TfR2 and unlike TfR1, the levels of the chimera did not respond to intracellular iron content. These results suggest that although holo-Tf binding to the ectodomain is necessary, the cytoplasmic domain of TfR2 is largely responsible for its stabilization by holo-Tf.

The on-line version of this article (available at http://www.jbc.org) contains supplemental material including Figs. 1–4.
FLAG epitope tag (DYKDDDDK) was added to the C terminus of TfR1 to distinguish the chimera from endogenous TfR1.

For the chimera of TfR1-cyto-tm/TfR2-ecto, a fragment of TfR1 cytoplasmic and transmembrane domain (aa 1–89) was amplified using primers of 5’-CTGGCGACCGCTTTGAGAATGT-3’ (forward) and 5’-TACCTGAGGCTTCCTGCGAC-3’ (TfR1 aa 85–89, TfR2 aa 105–109, reverse), a fragment of TfR2 ectodomain (aa 105–810) was amplified using primers of 5’-TTGGCTAATTGGGTAGGCTCCTGCGAC-3’ (TfR1 aa 85–89, TfR2 aa 105–109, forward) and 5’-CTGAAATTCCTCAGAAGTTTACATGT-3’ (reverse), then overlapping PCR was employed to generate TfR1-cyto-tm/TfR2-ecto using primers of 5’-CTGGCGACCGCTTTGAGAATGT-3’ (forward) and 5’-CTGAAATTCCTCAGAAGTTTACATGT-3’ (reverse).

The PCR product of TfR2-cyto/TfR1-tm-ecto-FLAG was digested with BamHI and EcoRI and inserted into pcDNA3 to generate pcDNA3-TfR2-cyto/TfR1-tm-ecto-FLAG, and the PCR product of TfR1-cyto-tm/TfR2-ecto was digested with HindIII and EcoRI and inserted into pcDNA3 to generate pcDNA3-TfR1-cyto-tm/TfR2-ecto. All constructs were verified by sequencing.

**Cell Culture**—Hep3B cells were maintained in minimal essential medium (Invitrogen) supplemented with 1.0 mM folic acid and 1% fetal bovine serum. Hep3B cells stably expressing TfR2-cyto/TfR1-tm-ecto-FLAG (Hep3B/TfR2CD) and wild type TfR2 (Hep3B/wtTfR2) were maintained in minimal essential medium with 400 μg/ml G418. For transient transfections, Hep3B cells in 6-well plates were transfected on day 1 with pcDNA3-TfR1-cyto-tm/TfR2-ecto using Lipofectamine (Invitrogen), and on day 3, cells were selected by 400 μg/ml G418 (Geneticin). The stable clone expressing TfR2-cyto/TfR1-tm-ecto-FLAG was recloned to ensure a pure cell line. Selected clones were screened by gel electrophoresis and Western blot analysis and immunofluorescence with M2 anti-FLAG antibody. Transfected cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and 400 μg/ml G418.

For transient transfections, Hep3B cells in 6-well plates were transfected on day 1 with pcDNA3-TfR1-cyto-tm/TfR2-ecto using Lipofectamine. On day 2, transfected cells in 1 well of a 6-well plate were split to 2 wells of a 12-well plate, on day 3, cells were treated with holo-Tf or PBS for 24 h, and on day 4 lysates were collected.

**Western Blots**—Cells were lysed on ice in NET-Triton X-100 buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.4, 1% Triton X-100) containing 1X Complete Mini Protease Inhibitor Mixture (Roche Diagnostics). Lysates were cleared by centrifugation at 15,000 g for 10 min. Protein concentration was measured using the BCA Protein Assay (Pierce). Aliquots of lysates containing 60 or 80 μg of total protein were incubated in 3.6X Laemml buffer (14) for 5 min at 95°C and subjected to reducing SDS-PAGE on 10% gels for analysis of TfR2-cyto/TfR1-tm-ecto-FLAG, TfR1-cyto-tm/TfR2-ecto, TfR1, TfR2, and actin or 12% gels for analysis of ferritin. After transfer to nitrocellulose, proteins were detected using M2 anti-FLAG (1:4,000), rabbit anti-TfR2 (1:10,000), 3B8 2A1 (1:5,000), H68.4 (1:10,000), mouse anti-actin (1:10,000) or rabbit anti-ferritin (1:2,000) primary antibodies and then horseradish peroxidase-conjugated (1:10,000) or fluorescently labeled (1:5,000) secondary antibodies. Bands were visualized by chemiluminescence (SuperSignal WestPico; Pierce) or were visualized and quantified by fluorescence imaging (Odyssey Infrared Imaging System; Li-Cor, Lincoln, NB). Rabbit anti-human TfR2 was incubated with Alexa 680 goat anti-rabbit fluorescent secondary antibody. All mouse monoclonal primary antibodies (M2 anti-FLAG, anti-actin, 3B8 2A1, and H68.4) were incubated with the IRDye 800 donkey anti-mouse fluorescent secondary antibodies. The fluorescent bands were converted to black and white images in the figures.

**Immunofluorescence**—Hep3B/TfR2CD cells and Hep3B cells transiently transfected with TfR1-cyto-tm/TfR2-ecto growing on coverslips were washed twice with PBS, fixed for 15 min with 4% (v/v) paraformaldehyde in PBS, then washed 3 times with PBS, and blocked with 10% fetal bovine serum in PBS for 60 min at room temperature. Cells were incubated in primary antibodies diluted in PBS containing 5% fetal bovine serum for 60 min, washed 3 X 5 min with PBS, incubated with secondary antibodies diluted in PBS for 60 min, and washed 3 X 5 min with PBS, and coverslips were mounted in ProLong Gold anti-fade reagent (Molecular Probes/Invitrogen). Images were acquired by
laser-scanning confocal microscopy using a Plan-Apochromat 20× objective on a Zeiss LSM 5 Pascal confocal inverted microscope. AlexaFluor 546 and AlexaFluor 488 signals were excited with helium neon (543 nm) and argon (488 nm) lasers, respectively, and obtained using the single-tracking function. Cells expressing TfR2-cyto/TfR1-tm-ecto-FLAG or TfR1-cyto-tm/TfR2-ecto were labeled with either 25 μg/ml M2 anti-FLAG or 8 μg/ml purified IgG fraction of the 16637 rabbit anti-TfR2 polyclonal anti-serum, respectively and visualized with either goat anti-mouse AlexaFluor 546 (1:500) or goat anti-rabbit AlexaFluor 488 (1:500), respectively.

**Immunoprecipitation**—Hep3B/TfR2CD (250 μg) and Hep3B (250 μg) (as a control) cell lysates were precleared with protein A or protein G for 30 min at 4 °C. The cell lysates were incubated overnight at 4 °C with either 2 μl of rabbit anti-FLAG to immunoprecipitate the chimera containing the FLAG epitope tag or in separate experiments with 2 μl of H68.4 to immunoprecipitate endogenous TfR1. After centrifugation, the pellets were washed 3 times for 5 min with NET-1% Triton X-100, and protein was eluted by boiling in SDS loading buffer at 95 °C for 5 min. The supernatant of immunoprecipitation with H68.4 was re-immunoprecipitated with 2 μl of H68.4 to determine how much endogenous TfR1 was left after the first immunoprecipitation. The majority (88%) of TfR1 was immunoprecipitated in the first round of immunoprecipitation, and the remaining 12% was immunoprecipitated in the second round. The first immunoprecipititation with rabbit anti-FLAG was split to two equal aliquots to detect the chimera with M2 anti-FLAG and endogenous TfR1 with H68.4, respectively. The sum of first and second immunoprecipititation of H68.4 detected with H68.4 is the total endogenous TfR1 level. All of these samples were subjected to 10% SDS-PAGE, transferred to nitrocellulose, and immuno-detected by H68.4 and M2 anti-FLAG and quantified by the IRDye 800 donkey anti-mouse fluorescent secondary antibodies using a Licor fluorimeter.

**RESULTS**

The **Cytoplasmic Domain of TfR2** Is Largely Responsible for the **Stabilization of TfR2** by Holo-Tf—To determine which domains are responsible for stabilization of TfR2 by holo-Tf, chimeras consisting of different combinations of the ecto-, transmembrane, and cytoplasmic domains of TfR1 and TfR2 were generated by overlapping PCR (Fig. 1A). Like TfR1, TfR2 is a type II transmembrane protein consisting of an N-terminal cytoplasmic domain, a single transmembrane domain, and a C-terminal ectodomain. A FLAG epitope tag was added to the C terminus of TfR1 in chimera TfR2cyto/TfR1-tm-ecto-FLAG to distinguish from endogenous TfR1 (Fig. 1A). Hep3B cells, which express endogenous TfR1 but no detectable TfR2, were used to express the chimera with the cytoplasmic domain of TfR2 and the transmembrane and ectodomains of TfR1. The chimera was stably expressed in Hep3B cells (Hep3B/TfR2CD). This chimera formed heterodimers with endogenous TfR1. Cell lysates of Hep3B/TfR2CD were immunoprecipitated with anti-FLAG antibody to isolate the TfR2cyto/TfR1-tm-ecto-FLAG chimera and TfR1 associated with the chimera. The amount of TfR1 co-precipitating with the chimera was compared with the total TfR1 immunoprecipitated (Fig. 1B). Quantitation of the

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**FIGURE 1. Response of TfR2-cyto/TfR1-tm-ecto-FLAG and TfR1-cyto-tm/TfR2-ecto to holo-Tf**. A, a schematic of chimeras consisting of the ecto-, transmembrane, and cytoplasmic domains of TfR1 and TfR2. B, TfR2-cyto/TfR1-tm-ecto-FLAG form heterodimers with endogenous TfR1. Cell lysates (250 μg) of Hep3B/TfR2CD or Hep3B (as a control) were immunoprecipitated (IP) with rabbit anti-FLAG (lanes 1–4) or H68.4 (lanes 5–6) as described under "Materials and Methods," subjected to 10% SDS-PAGE, transferred to nitrocellulose, and immunodetected with M2 anti-FLAG (lanes 1–2) or H68.4 (lanes 3–6). Bands were quantified with fluorescently labeled secondary antibodies and fluorimetry. W8, Western blot. C, the chimera of TfR2-cyto/TfR1-tm-ecto-FLAG traffics to the cell surface. Hep3B cells stably expressing TfR2-cyto/TfR1-tm-ecto-FLAG (Hep3B/TfR2CD) were seeded at 3 × 10^5/cm² in 12-well plate. The cells were fixed but not permeabilized, labeled with M2 anti-FLAG, and detected by goat anti-mouse AlexaFluor 546 as described under "Materials and Methods." The scale bar represents 10 μm. D, TfR2-cyto/TfR1-tm-ecto-FLAG increases by 2.6-fold after the addition of holo-Tf to the medium of Hep3B/TfR2CD. Hep3B stably expressing TfR2-cyto/TfR1-tm-ecto-FLAG (Hep3B/TfR2CD) were incubated in the medium containing 25 μM holo-Tf for 24 h. Lysates (60 μg) were subjected to SDS-PAGE and transferred to nitrocellulose. The chimera and actin were detected by M2 anti-FLAG and anti-actin, respectively, and quantitated via secondary fluorescent antibodies as described under "Materials and Methods." In control E, the chimera of TfR1-cyto-tm/TfR2-ecto traffics to the cell surface. Hep3B cells were seeded at 3 × 10^5/cm² on coverslip in 2-well plate and transient-transfected with pcDNA3/TfR1-cyto-tm/TfR2-ecto for 48 h. Fixed but not permeabilized cells were labeled with rabbit anti-human TfR2 (16637) and detected by goat anti-rabbit AlexaFluor 488 as described under "Materials and Methods." The scale bar represents 10 μm. F, TfR1-cyto-tm/TfR2-ecto remained unchanged after the addition of holo-Tf to the medium of the cells transient expressing TfR1-cyto-tm/TfR2-ecto. Hep3B cells transient transfected by pcDNA3-TfR1-cyto-tm/TfR2-ecto were treated with 25 μM holo-Tf for 24 h, and then chimera and actin were analyzed by Western blot detected with rabbit anti-human TfR2 and anti-actin, respectively, as described in C. Experiments in D and F were repeated six and two times, respectively, with similar results.
relative amounts of TfR1 immunoprecipitated demonstrated that ~7% of TfR1 formed heterodimers with TfR2cyto/TfR1-tm-ecto-FLAG. Thus, only a small fraction of TfR1 associated with this chimera. TfR2cyto/TfR1-tm-ecto-FLAG trafficked to the cell surface and bound holo-Tf, indicating that it was not malfolded and retained in the endoplasmic reticulum (Fig. 1C). To test whether the cytoplasmic domain was sufficient for the increase in the steady state levels of TfR2 by holo-Tf, Hep3B/TfR2CD cells were treated with 25 μM (2 mg/ml) holo-Tf for 24 h, and protein levels were examined by Western blot detection with M2 anti-FLAG antibody. The levels of the chimera increased by about 2.6-fold (Fig. 1D). This is the same- fold increase that was seen in HepG2 cells that endogenously express TfR2 (8) and Hep3B cells stably transfected with TfR2 (15), indicating that the cytoplasmic domain of TfR2 has an important role in its response to holo-Tf. Similar to TfR2, which was able to internalize anti-TfR2 antibody and partially localize in EEA1 (early endosome antigen 1)-positive compartments (supplemental Figs. 1–3), the chimera was capable of internalizing M2 anti-FLAG antibody (supplemental Fig. 4).

Hep3B cells were transiently transfected with a chimera consisting of the ectodomain of TfR2 and the remaining domains of TfR1 (TfR1cyto-tm/TfR2-ecto) to examine whether the ectodomain of TfR2 also plays a role in response to holo-Tf. This chimera also trafficked to the cell surface and bound holo-Tf (Fig. 1E), indicating that it was not malfolded. Cells were treated with 25 μM holo-Tf for 24 h, and protein levels were examined by Western blot analysis with rabbit anti-TfR2. The chimera with the ectodomain of TfR2 and the remaining domains of TfR1 did not respond to the addition of holo-Tf (Fig. 1F). Thus, the cytoplasmic domain of TfR2 is largely responsible for the stabilization of TfR2 by holo-Tf.

Holo-Tf Is Required for the Increased Level of TfR2cyto/TfR1-tm-ecto-FLAG—Holo-Tf supplies cells with both Tf and iron. In HepG2 cells that endogenously express TfR2, the binding of holo-Tf to TfR2 appeared to be necessary for the stabilization of TfR2 (8, 15). Neither the addition of apoTf, which does not bind to TfR2 appreciably at neutral pH, nor iron to cells increased TfR2 levels (8, 9). To test whether the same was true in cells expressing the chimera with the cytoplasmic domain of TfR2, Hep3B/TfR2CD cells were cultured in 25 μM apoTf for 24 h, and the levels of TfR2cyto/TfR1-tm-ecto-FLAG were quantified by Western blot detection with fluorescence-labeled secondary antibodies. Similar to TfR2 (8, 9), TfR2cyto/TfR1-tm-ecto-FLAG increased by about 2.6-fold in response to holo-Tf but remained unaltered in response to apoTf (Fig. 2A).

Holo-Tf Is Required for the Increased Level of TfR2cyto/TfR1-tm-ecto-FLAG—Holo-Tf supplies cells with both Tf and iron. In HepG2 cells that endogenously express TfR2, the binding of holo-Tf to TfR2 appeared to be necessary for the stabilization of TfR2 (8, 15). Neither the addition of apoTf, which does not bind to TfR2 appreciably at neutral pH, nor iron to cells increased TfR2 levels (8, 9). To test whether the same was true in cells expressing the chimera with the cytoplasmic domain of TfR2, Hep3B/TfR2CD cells were cultured in 25 μM apoTf for 24 h, and the levels of TfR2cyto/TfR1-tm-ecto-FLAG were quantified by Western blot detection with fluorescence-labeled secondary antibodies. Similar to TfR2 (8, 9), TfR2cyto/TfR1-tm-ecto-FLAG increased by about 2.6-fold in response to holo-Tf but remained unaltered in response to apoTf (Fig. 2A).
The Effect of Holo-Tf on TfR2-cyto/TfR1-tm-ecto-FLAG Is Time- and Dose-dependent—Hep3B/TfR2CD cells were cultured in medium with 25 μM holo-Tf for 0–72 h, and protein levels were examined by Western blot analysis to test time dependence of holo-Tf on stabilization of TfR2-cyto/TfR1-tm-ecto-FLAG. After the addition of 25 μM Tf, TfR2-cyto/TfR1-tm-ecto-FLAG levels increased visibly within 4 h and reached a maximum at 48 h (Fig. 4A). Control cells that were not treated with holo-Tf did not show significant changes in TfR2-cyto/TfR1-tm-ecto-FLAG (Fig. 4B). By comparison, in HepG2 cells, which endogenously express TfR2, increases in TfR2 can also be detected within 4 h after holo-Tf treatment and reach maximal levels at 48 h (8).

The dose response of TfR2-cyto/TfR1-tm-ecto-FLAG to holo-Tf was examined by adding 0–30 μM (0–2.4 mg/ml) holo-Tf to the medium for 24 h. Quantitative Western blot analysis showed that TfR2-cyto/TfR1-tm-ecto-FLAG increased as the concentration of holo-Tf increased from 0 to 30 μM (Fig. 5A). The increase in TfR2-cyto/TfR1-tm-ecto-FLAG was half-maximal when the concentration of holo-Tf was ~3.1 μM (Fig. 5B), which was consistent with the observations of Johnson and Enns (8) and Robb and Wessling-Resnick (9), indicating the chimera acts in the same way as TfR2. The chimera was stabilized maximally when the holo-Tf concentration was 30 μM. The affinity of TfR1 for holo-Tf is much higher than that of TfR2; however, the addition of the FLAG epitope tag to the C terminus of TfR1 decreased the affinity of Tf for TfR1 by about 10-fold (13) and made the chimera responsive to changes in holo-Tf over the same range in concentration as TfR2 (8).

Holo-Tf Increases TfR2-cyto/TfR1-tm-ecto-FLAG Protein Stability—The elevation in TfR2 is due to an increase of its half-life in cells exposed to holo-Tf (8, 9). To investigate
whether the elevation in TfR2-cyto/TfR1-tm-ecto-FLAG also results from an increase in stability of the chimera, we measured its half-life in Hep3B/TfR2CD cells. After culturing for 24 h in the medium without (0 μM) or with 25 μM holo-Tf, 100 μg/ml cycloheximide was added to the medium to inhibit protein synthesis. The intensity of each band was normalized to the intensity of actin and then normalized to that of the 0-h sample. A, the chimera containing the TR2 cytoplasmic domain increased gradually with time in Hep3B/TR2CD cells treated by holo-Tf. B, the chimera containing the TR2 cytoplasmic domain remained unchanged in Hep3B/TR2CD cells treated with PBS. The graphs show the mean relative amount of two experiments; error bars indicate deviation from the mean.

FIGURE 4. TfR2-cyto/TfR1-tm-ecto-FLAG increases in a time-dependent manner. Hep3B/TR2CD cells were cultured for 0–72 h after the addition of 25 μM holo-Tf or PBS to the medium. Chimera and actin from 60 μg of cell lysate were detected on Western blots with M2 anti-FLAG and anti-actin antibodies and quantified by fluorescence-labeled secondary. The intensity of each band was normalized to the intensity of actin and then normalized to that of the 0-h sample. A, the chimera containing the TR2 cytoplasmic domain increased gradually with time in Hep3B/TR2CD cells treated by holo-Tf. B, the chimera containing the TR2 cytoplasmic domain remained unchanged in Hep3B/TR2CD cells treated with PBS. The graphs show the mean relative amount of two experiments; error bars indicate deviation from the mean.

FIGURE 5. The response of TfR2-cyto/TfR1-tm-ecto-FLAG to holo-Tf is concentration-dependent. Hep3B/TR2CD cells were cultured for 24 h after the addition of 0–30 μM holo-Tf to the medium, and cell lysates (60 μg) were analyzed by Western blot (A) with M2 anti-FLAG and anti-actin and fluorescently labeled secondary antibodies for quantification. The intensity of each band was normalized to the intensity of actin and then normalized to that of the 0 μM Tf sample. The increase in TR2 is half-maximal at ~3.1 μM holo-Tf. B, the graph shows the mean relative amount of four experiments; error bars indicate average deviation from the mean.

FIGURE 6. Regulation of TfR2-cyto/TfR1-tm-ecto-FLAG occurs at the level of protein stability. Hep3B/TR2CD cells seeded at 3×10^4 cells/cm^2 were incubated in normal medium or medium with 25 μM holo-Tf for 24 h before the addition of 100 μg/ml cycloheximide for 0, 2, 4, 8, 12, and 24 h. Cells were solubilized, lysates from triplicate wells were pooled, and half of each sample was analyzed by Western blot. The chimera and actin were detected with M2 anti-FLAG and anti-actin, respectively, followed by fluorescently labeled antibodies for quantification. The integrated intensity of chimera was normalized to that of actin, which did not change detectably over the time course of the experiment. The normalized intensity was expressed as a percentage of the normalized intensity at time 0, and the log of this value was plotted. Half-life was determined by linear regression analysis. The graph shows the mean of five experiments; error bars indicate average deviation from the mean.

**TfR2 Stability and Tf**

A Monoclonal Antibody to the Ectodomain of TfR2 Downregulates rather than Stabilizes TfR2—To test whether binding of another protein to the ectodomain could also stabilize TfR2, we treated Hep3B cells stably expressing wild type TfR2 with a monoclonal antibody to the ectodomain of TfR2 (9F8 1C11) for dominant role in the trafficking of TfR2 in response to holo-Tf treatment of cells.

**FIGURE 4. TfR2-cyto/TfR1-tm-ecto-FLAG increases in a time-dependent manner.** Hep3B/TR2CD cells were cultured for 0–72 h after the addition of 25 μM holo-Tf or PBS to the medium. Chimera and actin from 60 μg of cell lysate were detected on Western blots with M2 anti-FLAG and anti-actin antibodies and quantified by fluorescence-labeled secondary. The intensity of each band was normalized to the intensity of actin and then normalized to that of the 0-h sample. A, the chimera containing the TR2 cytoplasmic domain increased gradually with time in Hep3B/TR2CD cells treated by holo-Tf. B, the chimera containing the TR2 cytoplasmic domain remained unchanged in Hep3B/TR2CD cells treated with PBS. The graphs show the mean relative amount of two experiments; error bars indicate deviation from the mean.
Iron is an essential but potentially noxious metal for almost all organisms (16). Therefore, iron uptake into cells and body is tightly regulated. In humans, iron deficiency results in anemia, whereas excess iron results in hemochromatosis. The consequences of iron overload include liver cirrhosis, hepatocellular carcinoma, diabetes, heart failure, arthritis, and hypogonadism (17–22). Hereditary hemochromatosis (HH) is the most common life threatening hereditary disease in people of northern European descent. Four types of HH have been identified clinically. HH types 1, 2A, 2B, and 3 are autosomal recessive diseases caused by mutations in the HH protein, HFE (23), hemjuvelin (24), hepcidin (25), and TfR2 (26), respectively. In contrast, HH type 4 is an autosomal dominant form caused by mutations in the iron exporter, ferroportin 1 (27, 28). The focus of our work presented here is TfR2, the cause of type 3 HH.

TfR2 has been proposed to sense iron levels in the body. The protein sequence similarity of TfR2 with TfR1 in conjunction with its ability to bind and internalize Tf (5) and its predominant hepatic expression lead to the initial proposal that TfR2 was responsible for the non-TfR1-mediated iron uptake observed more than 6 years ago (7). The finding that mutations in TfR2 lead to a recessive form of HH with iron accumulation in the liver leads to speculation that it might possess another function. The same phenotype of iron overload in the TfR2 knock-out mice confirmed that the mutations were due to a lack of function rather than a gain of function (29).

How TfR2 senses bodily iron levels, however, is not known. TfR2 does not respond to intracellular iron levels in contrast to TfR1. In keeping with this observation, it has no predicted iron-responsive elements in its mRNA and does not respond to iron loading of cells (2, 3, 5, 7). Earlier work showed that TfR2 levels rise with increased Tf saturation only in hepatic cell lines whether they express endogenous or are transfected with TfR2 (8). Others have noted the increased levels of TfR2 with Tf treatment in non-hepatic cell lines (30). The basis for these differences remains unresolved. The proposed mechanism for TfR2 sensing bodily iron levels is that the degree of iron saturation reflects bodily iron stores. Patients with the TfR2 form of HH have abnormally low hepcidin levels (31). Hepcidin is a hormone secreted by the liver that negatively regulates iron efflux from intestinal cells into the blood. TfR2 is proposed to positively regulate hepcidin levels by an unknown mechanism (32). Thus, increased bodily iron levels results in increased Tf saturation that, in turn, leads to increased TfR2 levels and increased hepcidin synthesis. As a result, iron uptake from the intestine would decrease. Animal studies have supported this hypothesis (9). Rats fed with an iron-deficient diet with low Tf saturation have lower liver TfR2 levels than rats fed with a high iron diet (9). Liver TfR2 is also lower in the hypotransferrinemic mouse, which carries a splice-site mutation in Tf that results in the loss of circulating Tf, supporting the role of holo-Tf in the regulation of TfR2 (9). These results highlight potentially important role for liver TfR2 as a sensor of holo-Tf concentration in the blood and raise the question as to how holo-Tf stabilizes TfR2.

In this study we examined the domain responsible for the stabilization of TfR2 by holo-Tf. We first constructed two chimeras consisting of TfR1/TfR2 cytoplasmic domain, transmembrane domain, and ectodomain. We then demonstrated that, in Hep3B cells, TfR2-cyo/TfR1-tm-ecto-FLAG protein levels increased in a dose- and time-dependent manner in response to physiologic levels of holo-Tf and that the half-life of the chimera increased from 8.1 to 21.8 h. These data demonstrate an adaptive response of TfR2-cyo/TfR1-tm-ecto-FLAG protein levels to reflect changes in the saturation state of iron binding by Tf and confirm that the chimera acts in the same way as TfR2, which was reported by Johnson and Enns (8) and Robb and Wessling-Resnick (9). Like TfR2, the iron status of the cell does not appear to affect stability of TfR2-cyo/TfR1-tm-ecto-FLAG. Rather, Tf binding and saturation affect the stability of
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the chimera. In contrast, the stability of the TfR1cyto-tm/TfR2-ecto chimera was not changed by holo-Tf, which clearly shows the importance of the cytoplasmic domain in the stabilization of TfR2 by holo-Tf.

Our results in conjunction with the findings of a recent study have started to define the mechanism by which the stability of TfR2 is controlled. TfR2 is degraded in the lysosome (15). Treatment of the cells with holo-Tf decreases the fraction of TfR2 localizing to late endosome and increases the fraction of TfR2 localizing to recycling endosome, indicating holo-Tf redirects the trafficking of TfR2 from degradation pathway to recycling pathway (15). These events are controlled by the binding of Tf to TfR2 and cytoplasmic signals.

The stability of a membrane protein is determined by any one of its domains. Polar residues within the transmembrane domain can result in increased degradation of receptors (33). Cytoplasmic domains of membrane proteins can direct them to a degradation pathway, and ligands binding to ectodomains can alter these signals (34). Our data suggest that the transmembrane domain of TfR2, although it contains polar residues, does not appear to be critical for stabilization of TfR2 by Tf binding because the chimera containing only the cytoplasmic domain of TfR2 was stabilized to the same extent as the wild type TfR2. TfR2 is unusual because its ligand, Tf, stabilizes the receptor, which is reflected by increased levels of TfR2. Most ligands upon binding induce the trafficking of their receptors to lysosomes or do not affect the half-life of the receptor. Ligand binding or artificial cross-linking of receptors by polyvalent antibodies can aggregate receptors and direct them to multivesicular bodies and lysosomal degradation (35, 36). In the case of TfR2, Tf binding could prevent aggregation of TfR2 via its ectodomain. We have ruled out this possibility, however, because the chimera containing the ecto- and transmembrane domains of TfR1 and the cytoplasmic domain of TfR2 behaves indistinguishably from TfR2.

The detailed mechanism by which holo-Tf binding to TfR2 stabilizes the protein remains to be determined. Here, using chimeras, we have ruled out a mechanism involving an aggregation of the receptor via its ectodomain. Tomographic x-ray imaging of TfR1 indicates that Tf binds to the domains of the receptor proximal to the membrane (37). Tf most likely binds to TfR2 in a similar manner based on the high conservation of amino acids critical for Tf binding (4). Among these residues, the RGD sequence, which is important for Tf binding to both receptors, appears to be critical (38–40). Tf binding to either the TfR1 or TfR2 ectodomain could exert a mechanical strain on the transmembrane in the acidic milieu of the endosome where Tf undergoes a large conformation change when it loses its iron. This signal could be transmitted to the cytoplasmic domain of TfR2 and redirect it from the degradation to the recycling pathway. Alternatively, the Tf-TfR2 complex could bind to other some transmembrane protein to redirect it from a degradation pathway to a recycling pathway. These speculations lead to testable hypothesis for future work.

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REFERENCES

1. Kawabata, H., Nakamaki, T., Ikonomi, P., Smith, R. D., Germain, R. S., and Koeffler, H. P. (2001) Blood 98, 2714–2719
2. Fleming, R. E., Migas, M. C., Holden, C. C., Waheed, A., Britton, R. S., Tomatsu, S., Bacon, B. R., and Sly, W. S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2214–2219
3. Kawabata, H., Germain, R. S., Ikezoe, T., Tong, X., Green, E. M., Gombart, A. F., and Koeffler, H. P. (2001) Blood 98, 1949–1954
4. West, A. P., Jr., Bennett, M. J., Sellers, V. M., Andrew, N. C., Enns, C. A., and Bjorkman, P. J. (2000) J. Biol. Chem. 275, 38135–38138
5. Kawabata, H., Yang, R., Hiraoka, M., Kuwano, S., Gombart, A. F., and Koeffler, H. P. (1999) J. Biol. Chem. 274, 20826–20832
6. Klausner, R. D., Rouault, T. A., and Harford, J. B. (1993) Cell 72, 19–28
7. Kawabata, H., Germain, R. S., Kuwano, S., Takata, T., Said, J. W., and Koeffler, H. P. (2000) J. Biol. Chem. 275, 16618–16625
8. Johnson, M. B., and Enns, C. A. (2004) Blood 104, 4287–4293
9. Robb, A., and Wessling-Resnick, M. (2004) Blood 104, 4294–4299
10. Ajoka, R. S., and Kaplan, J. I. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6445–6449
11. Carpenter, G., and Cohen, S. (1976) J. Cell Biol. 71, 159–171
12. Vogt, T., Blackwell, A., Giannetti, A., Bjorkman, P., and Enns, C. (2002) Blood 101, 2008–2014
13. Warren, R. A., Green, F. A., and Enns, C. A. (1997) Mol. Biol. Cell., in press
14. Laemmli, U. K. (1970) Nature 227, 680–685
15. Johnson, M. B., Chen, J., Murchison, N., Green, F., and Enns, C. A. (2007) Mol. Biol. Cell., in press
16. Syed, B. A., Sargent, P. J., Faruq, S., and Evans, R. W. (2006) Hemoglobin 30, 69–80
17. Andrews, N. C. (2000)Curr. Opin. Pediatr. 12, 596–602
18. Cartwright, G. E., Edwards, C. Q., Kravitz, K., Skolnick, M., Amos, D. B., Johnson, A. N., and Buskjaer, L. (1979) N. Engl. J. Med. 301, 175–179
19. Cox, T. M., and Lord, D. K. (1989) Eur. J. Haematol. 42, 113–125
20. Andrews, N. C. (2000) Nat. Rev. Genet. 1, 208–217
21. Edwards, C. Q., Griffen, L. M., Goldgar, D., Drummond, C., Skolnick, M. H., and Kushner, J. P. (1988) N. Engl. J. Med. 318, 1355–1362
22. Bacon, B. R., Powell, L. W., Adams, P. C., Kresina, T. F., and Hoofnagle, J. H. (1999) Gastroenterology 116, 193–207
23. Feder, J. N., Giniere, A., Thomas, W., Tsuchihashi, Z., Ruddy, D. A., Basava, A., Dormishian, F., Domingo, R. J., Ellis, M. C., Fullan, A., Hinton, L. M., Jones, N. L., Kimmel, B. E., Kornblatt, S. G., Lauer, P., Lee, V. K., Loeb, D. B., Mapi, F. A., McClelland, E., Meyer, N. C., Mjint, G. A., Moeller, N., Moore, T., Morikang, E., Prasss, C. E., Quintana, L., Starnes, S. M., Schatzman, R. C., Brunke, K. J., Drayna, D. T., Risch, N. J., Bacon, B. R., and Wolf, R. K. (1996) Nat. Genet. 13, 399–408
24. Papanikolaou, G., Samuels, M. E., Ludwig, E. H., MacDonald, M. L., Franchini, P. L., Dube, M. P., Andres, L., McFarlane, J., Sakellaropoulos, N., Politou, M., Nemeth, E., Thompson, J., Risler, J. K., Zaborowska, C., Babakaiff, R., Radosmki, C. C., Pape, T. D., Daividas, O., Christakis, J., Brissot, P., Lockitch, G., Ganz, T., Hayden, M. R., and Goldberg, Y. P. (2004) Nat. Genet. 36, 77–82
25. Roetto, A., Papanikolaou, G., Politou, M., Alberti, F., Girelli, D., Christakis, J., Loukopoulos, D., and Camaschella, C. (2003) Nat. Genet. 33, 21–22
26. Camaschella, C., Roetto, A., Cali, A., De Gobbi, M., Garozzo, G., Carella, M., Majorano, N., Totaro, A., and Gasparini, P. (2000) Nat. Genet. 25, 14–15
27. Donovan, A., Brownlie, A., Zhou, Y., Shepard, J., Pratt, S. J., Moyer, J., Paw, B. H., Dreier, B., Barut, Z., Zupata, A., Law, T. C., Brugnara, C., Lux, S. E., Pinkus, G. S., Pinkus, J. L., Kingsley, P. D., Pals, J., Fleming, M. D., Andrews, N. C., and Zon, L. I. (2000) Nature 403, 776–781
28. Wallace, D. F., Pedersen, P., Dixon, J. L., Stephenson, P., Searle, J., Powell, L. W., and Subramaniam, V. N. (2002) Blood 100, 692–694
29. Wallace, D. F., Summerville, L., Lusby, P. E., and Subramaniam, V. N. (2005) Gut 54, 980–986
30. Deaglio, S., Capobianco, A., Cali, A., Bellora, F., Alberti, F., Righi, L., Sapino, A., Camaschella, C., and Malavasi, F. (2002) Blood 100, 3782–3789
31. Nemeth, E., Roetto, A., Garozzo, G., Ganz, T., and Camaschella, C. (2005) Blood 105, 1803–1806
32. Kawabata, H., Fleming, R. E., Gui, D., Moon, S. Y., Saitoh, T., O’Kelly, I., Umehara, Y., Wano, Y., Said, J. W., and Koeffler, H. P. (2005) Blood 105, 376–381
33. Zaliauskiene, L., Kang, S., Brouillette, C. G., Lebowitz, J., Arani, R. B., and Collawn, J. F. (2000) Mol. Biol. Cell 11, 2643–2655
34. Wiley, H. S. (2003) Exp. Cell Res. 284, 78–88
35. Furuno, K., Yano, S., Akasaki, K., Tanaka, Y., Yamaguchi, Y., Tsuji, H., Himeno, M., and Kato, K. (1989) J. Biochem. (Tokyo) 106, 717–722
36. Mellman, I., and Plutner, H. (1984) J. Cell Biol. 98, 1170–1177
37. Cheng, Y., Zak, O., Aisen, P., Harrison, S. C., and Walz, T. (2004) Cell 116, 565–576
38. Kawabata, H., Tong, X., Kawanami, T., Wano, Y., Hirose, Y., Sugai, S., and Koeffler, H. P. (2004) Br. J. Haematol. 127, 464–473
39. Dubljevic, V., Sali, A., and Goding, J. W. (1999) Biochem. J. 341, 11–14
40. Giannetti, A. M., Snow, P. M., Zak, O., and Bjorkman, P. J. (2003) PLoS Biol. 1, 341–350