Transferrin Receptor 2: Evidence for Ligand-induced Stabilization and Redirection to a Recycling Pathway

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Transferrin receptor 2 (TfR2) is a homologue of transferrin receptor 1 (TfR1), the protein that delivers iron to cells through receptor-mediated endocytosis of diferric transferrin (Fe₂Tf). TfR2 also binds Fe₂Tf, but it seems to function primarily in the regulation of systemic iron homeostasis. In contrast to TfR1, the trafficking of TfR2 within the cell has not been extensively characterized. Previously, we showed that Fe₂Tf increases TfR2 stability, suggesting that trafficking of TfR2 may be regulated by interaction with its ligand. In the present study, therefore, we sought to identify the mode of TfR2 degradation, to characterize TfR2 trafficking, and to determine how Fe₂Tf stabilizes TfR2. Stabilization of TfR2 by bafilomycin implies that TfR2 traffics to the lysosome for degradation. Confocal microscopy reveals that treatment of cells with Fe₂Tf increases the fraction of TfR2 localizing to recycling endosomes and decreases the fraction of TfR2 localizing to late endosomes. Mutational analysis of TfR2 shows that the mutation G679A, which blocks TfR2 binding to Fe₂Tf, increases the rate of receptor turnover and prevents stabilization by Fe₂Tf, indicating a direct role of Fe₂Tf in TfR2 stabilization. The mutation Y23A in the cytoplasmic domain of TfR2 inhibits its internalization and degradation, implicating YQRV as an endocytic motif.

INTRODUCTION

A truncation mutant of transferrin receptor 2 (TfR2), TfR2/Y250X, causes a rare form of hereditary hemochromatosis (type 3, HFE3), an iron overload disorder characterized by excess absorption of dietary iron and consequent deposition of iron in liver and other parenchymal tissues (Camaschella *et al.*, 1999, 2000). The analogous mutation or knockout of *Trfr2* in mice reproduces the disease phenotype (Fleming *et al.*, 2002; Wallace *et al.*, 2005). Thus, TfR2 is required for normal iron homeostasis.

TfR2, cloned in 1999, is a homologue of transferrin receptor 1 (TfR1; Kawabata *et al.*, 1999). The extracellular domains of the two receptors are 45% identical and 67% similar. TfR1 functions to deliver iron to cells through receptor-mediated endocytosis of its ligand transferrin (Tf), a serum protein that transports iron (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). On the cell surface, TfR1 binds iron-saturated transferrin (Fe₂Tf) at slightly basic pH. Fe₂Tf-TfR1 then internalizes in clathrin-coated vesicles to early endosomes.

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Abbreviations used: Ab, antibody; AP, adaptor protein; EEA, early endosomal antigen; FBS, fetal bovine serum; Fe₂Tf, diferric transferrin; HBSS, Hanks' balanced salt solution; LAMP, lysosome-associated membrane protein; MEM, minimal essential medium; PBS, phosphate-buffered saline; Tf, transferrin; TfR, transferrin receptor; Trfr2, murine transferrin receptor 2; TGN, trans-Golgi network; WT, wild-type; YXXØ, tyrosine (Y)-based endocytic motif, where X represents any amino acid and Ø represents a bulky hydrophobic residue.

In the acidic pH of early endosomes, iron releases from Tf, whereas Tf remains bound to TfR1. The complex then recycles, from either early or recycling endosomes, to the cell surface. At the slightly basic pH of the cell surface, TfR1 releases unsaturated Tf (apoTf) and again binds Fe₂Tf. TfR1 expression is ubiquitous, consistent with its role in cellular iron delivery. The stability of TfR1 mRNA is negatively regulated by intracellular iron levels through iron-responsive elements (IREs) in the 3' untranslated region (Mattia *et al.*, 1984; Ward *et al.*, 1984; Rao *et al.*, 1985; Sciort *et al.*, 1987; Owen and Kuhn, 1987; Casey *et al.*, 1988; Mullner and Kuhn, 1988; Lu *et al.*, 1989; Mullner *et al.*, 1989).

TfR2 differs from TfR1 in notable ways. TfR2 binds Tf in a pH-dependent manner, but its affinity for Fe₂Tf ($K_D \sim 30$ nM; Kawabata *et al.*, 2000; West *et al.*, 2000) is significantly lower than that of TfR1 ($K_D \sim 1$ nM, Tsunoo and Sussman, 1983; Enns *et al.*, 1991; Richardson and Ponka, 1997). Unlike TfR1, TfR2 expression is limited predominantly to hepatocytes (Kawabata *et al.*, 1999; Fleming *et al.*, 2000, 2002; Vogt *et al.*, 2003; Calzolari *et al.*, 2004; Zhang *et al.*, 2004) and is not regulated by intracellular iron (Fleming *et al.*, 2000; Kawabata *et al.*, 2000, 2001). TfR2 cannot compensate for TfR1, whose knockout in mice results in embryonic lethality due to severe anemia (Levy *et al.*, 1999). Because *Trfr2* mutation or knockout results in iron overload, TfR2 appears to function, not principally in cellular iron uptake and delivery, but rather in systemic iron homeostasis. The exact function of TfR2, however, is not known.

To investigate the function of TfR2, we previously characterized the response of TfR2 to Fe₂Tf in a human hepatoma cell line, HepG2, that endogenously expresses TfR2. Whereas ligand-receptor interactions frequently result in receptor down-regulation, addition of Fe₂Tf to the medium of HepG2 cells increases TfR2 by extending the half-life of

TfR2 from 4 to 14 h (Johnson and Enns, 2004). Interestingly, regulation of TfR2 by its ligand has only been observed in hepatoma cell lines (Johnson and Enns, 2004; Robb and Wessling-Resnick, 2004), suggesting the mechanism involves proteins, compartments, or pathways specific to the hepatocyte. Moreover, TfR2 regulation observed in HepG2 cells seems to recapitulate physiological regulation. Robb and Wessling-Resnick (2004) showed that TfR2 levels are elevated in mice with high serum transferrin saturation and reduced in mice with low serum transferrin saturation. In HepG2 cells, the response to Fe₂Tf was half-maximal at ~1–3 μ M Fe₂Tf, a physiologically relevant concentration range (Johnson and Enns, 2004; Robb and Wessling-Resnick, 2004).

The stabilization of TfR2 by Fe₂Tf suggests that the trafficking of this receptor may be regulated by its ligand. To test this hypothesis, we characterized the effect of Fe₂Tf and mutations on TfR2 localization and stabilization in two human hepatoma cell lines, HepG2 and Hep3B. We demonstrate that Fe₂Tf directs TfR2 from a degradative pathway to a recycling pathway, establish that direct interaction of TfR2 with Fe₂Tf stabilizes TfR2, and identify an endocytic motif in the intracellular domain of TfR2 necessary for TfR2 internalization and regulation.

MATERIALS AND METHODS

Reagents and Antibodies

Bovine serum albumin was obtained from Interger (Burlington, MA). Bafilomycin, cycloheximide, poly-L-lysine, ovalbumin, and saponin were obtained from Sigma-Aldrich (St. Louis, MO). Paraformaldehyde was from Electron Microscopy Sciences (Hatfield, PA). EasyTag Express Protein Labeling Mix containing [³⁵S]cysteine/methionine was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Generation of anti-TfR1 monoclonal antibody (mAb) 3B82A1, anti-TfR2 mAb 9F81C11, and anti-TfR2 rabbit serum was described previously (Vogt *et al.*, 2003; Johnson and Enns, 2004). H4A3 anti-LAMP1 ascites, developed by J. T. August and J.E.K. Hildreth, was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) under the auspices of the National Institute of Child Health and Human Development. Other primary antibodies were obtained from the following companies: mouse anti- β -actin, mouse anti- γ -adaplin, and rabbit anti-Rab7 were from Sigma-Aldrich (St. Louis, MO); mouse anti-early endosome antigen (EEA1) was from Abcam (Cambridge, United Kingdom); mouse anti-Golgin97 was from Invitrogen (Carlsbad, CA); and H68.4 mouse anti-TfR1 was from Zymed Laboratories (South San Francisco, CA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Chemicon International (Temecula, CA). Fluorescently labeled Alexa 488, Alexa 543, and Alexa 680 secondary antibodies were from Invitrogen. Fluorescently labeled IRDye 800 secondary antibody was from Rockland (Gilbertsville, PA).

Constructs

Full-length *TfR2* transcript was amplified by polymerase chain reaction (PCR) from HepG2 cDNA by using the forward primer 5'-gaattcgaggcttcaggagg-gacacaagcatg-3' and the reverse primer 5'-gcggccgcggcttattgatagcttg-3', designed to introduce flanking EcoRI and NotI restriction sites, respectively. The PCR product was cloned into a pGemT (Promega, Madison, WI) vector and subcloned into a pcDNA3.1+ /Neo vector (Invitrogen). Mutations were introduced by site-directed mutagenesis by using the QuikChange XL kit (Stratagene, La Jolla, CA).

Cell Culture

HepG2 and Hep3B human hepatoma cells obtained from American Type Culture Collection (Manassas, VA) were cultured in minimal essential medium (MEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1.0 mM sodium pyruvate, and 0.1 mM nonessential amino acids (Invitrogen). For metabolic labeling, cells were washed twice with phosphate-buffered saline (PBS) and incubated in labeling medium (MEM without L-methionine or L-glutamine (PromoCell, Heidelberg, Germany) supplemented with 10% FBS, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 100 μ M [³⁵S]cysteine/methionine) without or with 25 μ M Fe₂Tf for the indicated times at 37°C.

Transfection

Hep3B cells, seeded at 3.1×10^4 cells/cm² 16 h earlier, were transfected in Opti-MEM (Invitrogen) by using Lipofectamine (Invitrogen) according to the

manufacturer's instructions, 0.2 μ g/cm² plasmid, and a Lipofectamine/DNA ratio of 2.5 (microliters per microgram). Normal medium was replaced 4 h later. For stable transfections in six-well plates, cells were split to four (100-mm) dishes 3 d later and selected with 400 μ g/ml Geneticin (G-418) (Calbiochem, San Diego, CA). Colonies were picked after 2 wk. For transient transfections in 60-mm dishes, cells were split to six (4-mm) wells 30 h later, cultured for 16 h, and then cultured for an additional 24 h in the absence or presence of 25 μ M Fe₂Tf. This approach was found to minimize fluctuations in expression level that result from variations in transient transfection efficiency.

SDS-PAGE and Western Blot

Cells were lysed in NETT (150 mM NaCl, 5 mM EDTA [EDTA], and 10 mM Tris base, pH 7.4 with 1.0% [vol/vol] Triton X-100) with 1X Complete Mini Protease Inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) on ice for 15 min. Lysates were collected and cleared by centrifugation at 5000 $\times$ g for 15 min. Total protein concentration was measured by bicinchoninic acid assay (Pierce Chemical, Rockford, IL). Samples containing 10–20 μ g of total protein were diluted into 4X Laemmli buffer, heated to 95°C for 5 min, loaded on 10% denaturing gels, and analyzed by SDS-PAGE, followed by Western blot with HRP-conjugated or fluorescence-conjugated secondary antibodies as described previously (Johnson and Enns, 2004).

Immunofluorescence

The subcellular localization of TfR2 was assessed by double-labeling immunofluorescent detection. For colocalization with Rab7, TfR2 was detected using the purified IgG fraction of the 9F81C11 mouse anti-TfR2 supernatant (4.8 μ g/ml), and Rab7 was detected with a rabbit polyclonal antibody (1:1000). For colocalization with all other markers, TfR2 was detected using the purified IgG fraction of the 16637 rabbit anti-TfR2 polyclonal anti-serum (8 μ g/ml). Established markers of other intracellular compartments were detected with various mouse monoclonal antibodies as follows: TfR1 (3B82A1 at 1.5 μ g/ml, H68.4 at 1:500), EEA1 (1:100), adaptor protein (AP)-1 (1:100), Golgin97 (1:125). Rabbit polyclonal antibodies were detected with goat anti-rabbit Alexa Fluor 488 (1:500). Mouse monoclonal antibodies were detected with goat anti-mouse Alexa Fluor 543 (1:500).

For colocalization with Rab7, cells were rinsed twice with wash buffer (1.8 mM calcium chloride, 2.5 mM magnesium acetate, 75 mM potassium acetate, and 25 mM HEPES, pH 7.2), permeabilized, and extracted with permeabilization buffer (0.1% saponin [wt/vol] and 0.1% bovine serum albumin [wt/vol] in wash buffer) for 30 min at room temperature (RT), rinsed twice with wash buffer, fixed in 2% (vol/vol) paraformaldehyde in PBS for 30 min at RT, rinsed twice with wash buffer, and quenched with 10 mM glycine in wash buffer for 10 min at RT. All subsequent dilutions and washes were done with permeabilization buffer to maintain cell permeabilization. Cells were incubated in primary antibodies for 30 min, washed three times for 5 min, incubated with secondary antibodies for 30 min, and washed five times for 5 min. Coverslips were rinsed an additional three times in wash buffer and two times in distilled deionized water before mounting.

For colocalization of TfR2 with all other markers, cells were washed twice in Hanks' balanced salt solution (HBSS; Sigma-Aldrich), fixed for 15 min with 4% (vol/vol) paraformaldehyde in HBSS, quenched for 10 min in 10 mM glycine in HBSS, permeabilized for 10 min with 0.2% Triton-X 100 in HBSS, and blocked with 3% bovine serum albumin (BSA) in HBSS for 30 min at room temperature. Cells were incubated in primary antibodies, diluted into 3% BSA in HBSS, for 30 min, washed three times for 5 min with HBSS, incubated with secondary antibodies diluted in 3% BSA in HBSS for 30 min, washed five times for 5 min with HBSS, and rinsed twice with distilled deionized water. Where indicated, nuclei were stained by addition of ToPro3 (1:1000; Invitrogen) to the secondary antibody incubation. Coverslips were mounted in ProLong Gold anti-fade reagent (Invitrogen).

Confocal Microscopy

Images were acquired by laser-scanning confocal microscopy using the Zeiss X100/1.45 numerical aperture oil immersion objective lens (α Plan-Fluar) on a Zeiss LSM 5 Pascal confocal inverted microscope. Alexa Fluor 543 and Alexa Fluor 488 signals were sequentially excited with helium neon (543-nm) and argon (488-nm) lasers, respectively, and obtained using the multitracking function. Colocalization was quantified using the colocalization module in Pascal. After correcting for background in each image, colocalization was assessed as the fraction of TfR2 pixels colocalizing with TfR1, EEA1, Golgin97, AP-1, or Rab7 pixels.

Immunoprecipitation of ³⁵S-labeled TfR2

At the indicated times, cells were placed on ice, washed two times with ice-cold PBS, and lysed in NETT with 1 mg/ml ovalbumin. Lysates were precleared with 50 μ l of Pansorbin for 1 h at 4°C. Pansorbin was pelleted by centrifugation for 2 min at 13,000 $\times$ g. The cleared lysate was then transferred to a tube containing 2.5 μ l of 16637 rabbit anti-TfR2 antiserum prebound to 50 μ l of Pansorbin and immunoprecipitated for 1 h at 4°C. The sample was pelleted, resuspended in 100 μ l of NETT/ovalbumin, and washed through

NETT/ovalbumin containing 15% sucrose (wt/vol). Sample was resuspended in 50 μ l of 2X Laemmli buffer (Laemmli, 1970), heated at 95°C under reducing conditions for 5 min, centrifuged at $13,000 \times g$ for 2 min to pellet Pansorbin, and subjected to SDS-PAGE (10% polyacrylamide). The gel was then dried and exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) for quantification and film for image acquisition.

Rates of Endocytosis of Wild-Type (WT), Y23A, and G679A Tfr2

Hep3B cells, seeded at 2.8×10^4 cells/cm² 16 h earlier in 10-cm plates, were transfected in Opti-MEM (Invitrogen) by using 12 μ g of WT, Y23A, or G679A Tfr2 plasmid and 36 μ l of Lipofectamine (Invitrogen) according to the manufacturer's instructions. Medium (MEM and 20% FBS) was added to the plates 6 h later and replaced 24 h later by growth medium. Forty-eight hours after transfection, both the plasmid-transfected and mock-transfected Hep3B cells were washed twice with ice-cold PBS and then detached from 10-cm dishes by using cell dissociation buffer (Invitrogen) for 10 min at 37°C. Cells were collected and divided into five tubes and pelleted by centrifugation at 1500 rpm for 5 min. Cells were incubated in 25 μ g/ml purified rabbit anti-Tfr2 diluted in ice-cold fluorescence-activated cell sorting staining buffer (FSB) composed of Hanks' balanced salt solution, no Ca²⁺, Mg²⁺, or phenol red, 10 mM HEPES, pH 7.4, and 1% FBS on ice for 30 min, after which they were washed by underlaying with FBS and centrifugation. Cells were transferred to 37°C assay medium for 0, 4, 8, 12, and 16 min to allow the internalization of the prebound antibody before fixing in cold 4% paraformaldehyde-PBS for 20 min on ice. The fixed cells were washed once with cold FSB and the remaining uninternalized antibody was detected with an Alexa Fluor 488 goat anti-rabbit secondary antibody (1:600 dilution in FSB) by incubating 30 min on ice followed by washing with cold FBS. The amount of fluorescence was quantified by fluorescence flow cytometry BD Biosciences FACSCalibur flow cytometer. Profiles were gated on intact cells based on morphology, and arithmetic mean fluorescent intensity, at each time t. The Hep3B mock-transfected control was subtracted from the Tfr2-expressing cells. The control signal was sixfold less than the Tfr2/G679A-expressing cells.

RESULTS

Tfr2 Undergoes Lysosomal Degradation

Fe₂Tf increases the half-life of Tfr2 from 4 to 14 h in HepG2 cells (Johnson and Enns, 2004), indicating that Tfr2 degradation is a regulated process. To further understand this process, we set out to determine whether Tfr2 degradation occurs in the lysosome. Lysosomal degradation of Tfr2 was assessed by treating HepG2 cells with bafilomycin, an inhibitor of the vacuolar H⁺-ATPase (Bowman *et al.*, 1988), to dissipate the endosomal pH gradient and thereby block transport to lysosomes (van Weert *et al.*, 1995). Cells were treated in the absence and presence of cycloheximide, an inhibitor of protein synthesis, to prevent further Tfr2 synthesis. The presence of bafilomycin resulted in a significant increase in Tfr2 protein (Figure 1). Consistent with previous results, Tfr2 decreased significantly over a 4 h-time course in cells treated with cycloheximide. In these cells, the addition of bafilomycin attenuated this effect, restoring the Tfr2 level to that in control cells, a result in keeping with lysosomal degradation of Tfr2. HepG2 cells were treated with ALLN and MG-132 to inhibit proteasome activity to assess the role of the proteasome in Tfr2 degradation. To control for the inhibition of calpains and cathepsins by ALLN, cells were also treated with ALLM, which inhibits calpains and cathepsins, but not the proteasome. Inhibition of proteasome activity did not significantly alter level of Tfr2 (Figure 1C).

Subcellular Localization of Tfr2

Based on our results, we hypothesized that Fe₂Tf prevents lysosomal degradation of Tfr2 by diverting Tfr2 from a degradative pathway and predicted that Fe₂Tf would alter the subcellular localization of Tfr2. Because the subcellular localization of Tfr2 has not been fully described, we first characterized its intracellular trafficking by examining its colocalization with various established markers of subcellular compartments. Antibodies against EEA1, Tfr1, and Rab7 were used to label early (Mu *et al.*, 1995), early/recycling,

Figure 1. Bafilomycin blocks Tfr2 degradation in HepG2 cells. HepG2 cells were seeded at 2.5×10^4 cells/cm² in 12-well plates 4 d before treatment. (A and B) Cells were treated with 50 μ M bafilomycin (Baf) in the presence or absence of 100 μ g/ml cycloheximide (CHX) for 4 h. (A) Lysates (20 μ g of total protein) were separated by SDS-PAGE and immunoblotted with anti-Tfr2 serum and anti- β -actin antibody followed by fluorescence-labeled secondary antibodies (all 1:10,000). (B) The intensities of Tfr2 bands were normalized to the intensities of β -actin bands and expressed relative to untreated cells (control). Graph represents data averaged from four independent experiments. p values of 0.01–0.05, 0.01–0.001, and <0.001 indicate changes that are significant (*), very significant (**), and extremely significant (***), respectively, when evaluated by Student's *t* test. (C) Cells were treated with 100 μ M ALLM, 100 μ M ALLN, or 25 μ M MG-132 as indicated for 4 h. Lysates were analyzed as described for A. ALLM, ALLN, and MG-132 did not significantly alter Tfr2 levels in three independent experiments.

and late endosomal (Chavrier *et al.*, 1990) populations (Figure 2, A, D, and G), respectively. Confocal microscopy analysis showed that Tfr2 was present in punctate structures in the perinuclear region and throughout the cell periphery (Figure 2). Tfr2 partially colocalized with all three endosomal markers (Figure 2, C, F, and I), indicating that Tfr2 traffics through endocytic, recycling, and degradative pathways.

Although results show that Tfr2 degradation occurs in the lysosome (Figure 1), we observed very little colocalization of Tfr2 with the lysosomal marker lysosome-associated membrane protein (LAMP)-1 by immunofluorescence (Figure 2, M–O). The lack of colocalization between Tfr2 and LAMP-1 is likely due to rapid degradation of Tfr2 within the lysosome. These results are consistent with the lack of degradation intermediates seen in Western blots and immu-

Figure 2. Subcellular localization of TfR2. (A–U) TfR2 localizes to endosomes and the *trans*-Golgi network. HepG2 cells were seeded at 6.25×10^3 cells/cm² on poly-L-lysine-treated glass coverslips and cultured for 2 d before fixation, permeabilization, and labeling as described in *Materials and Methods*. Indicated markers are shown in magenta (A, D, G, J, M, P, and S), TfR2 is shown in green (B, E, H, K, N, Q, and T), and colocalization is shown in white (C, F, I, L, O, R, and U). Bar, 5 μm (unless otherwise indicated).

noprecipitations of TfR2 from HepG2 cells (Johnson and Enns, 2004).

We also examined the colocalization of TfR2 with markers of the *trans*-Golgi network (TGN). Membrane proteins reach the TGN during biosynthesis, and, in some cases, after internalization from the plasma membrane (Snider and Rogers, 1985; Stoorvogel *et al.*, 1988). TfR2 was observed to colocalize with TGN marker Golgin97 in the perinuclear region of cells (Figure 2R). Colocalization of TfR2 with AP-1 (Figure 2U), which facilitates vesicle transport between endosomes and the TGN and localizes to both compartments, was also observed. TfR2 and AP-1 colocalization was predominantly in the perinuclear region (Figure 2U) and only occasionally detectable in peripheral vesicles (data not shown).

Diferic Tf Regulates the Subcellular Localization of TfR2

The subcellular localization of TfR2 is consistent with that of a membrane protein trafficking through biosynthetic, recy-

cling, and degradative pathways (Figure 2). Because Fe₂Tf stabilizes TfR2, we predicted that the fraction of TfR2 localizing to recycling endosomes might increase in cells treated with Fe₂Tf. Quantitative colocalization analysis was used to measure the colocalization of TfR2 with EEA1, TfR1, Rab7, Golgin97, or AP-1 in HepG2 cells untreated or treated with Fe₂Tf (Figure 3A). No difference in the fraction of TfR2 colocalizing with EEA1, Golgin97, or AP-1 was detected. The colocalization of TfR2 with TfR1 increased from 0.42 ± 0.028 in untreated cells to 0.51 ± 0.022 in Fe₂Tf-treated cells. Because no increase in the colocalization of TfR2 with the early endosome marker EEA1 was detected, we interpret the increase in colocalization of TfR2 with the early/recycling endosome marker TfR1 as an increase in TfR2 localization to recycling endosomes. The increase in TfR2 colocalization to recycling endosomes was accompanied by a decrease in TfR2 colocalization with Rab7 in late endosomes, from 0.21 ± 0.015 in untreated cells to 0.17 ± 0.009 in Fe₂Tf-treated cells. Together, these results suggest that Fe₂Tf redi-

Figure 3. Fe₂Tf regulates the subcellular localization of Tfr2. (A) Fe₂Tf alters the trafficking of Tfr2. Before the experiment, HepG2 cells were incubated for 48 h without or with 25 μ M Fe₂Tf. As in Figure 2, cells were double labeled and visualized by scanning confocal microscopy. The effect of Fe₂Tf on the subcellular localization of Tfr2 was assessed by quantitative colocalization analysis. The fraction of Tfr2 signal colocalizing with EEA1, Tfr1, Rab7, Golgin97, or AP-1 signal was analyzed in 20–40 images per condition acquired in two to three independent experiments. Data were evaluated by Student's *t* test. *p* values of 0.01–0.05 and 0.01–0.001 indicate changes that are significant (*) and very significant (**), respectively. (B and C) Fe₂Tf does not retain Tfr2 at the cell surface. The distribution of Tfr2 in HepG2 cells was assessed by differential immunoprecipitation. HepG2 cells, seeded at 2.5×10^4 cells/cm² in 35-mm wells, were cultured in medium without or with 25 μ M Fe₂Tf for 48 h. Cells were then placed on ice, and culture medium was replaced with 1 ml of MEM containing 10% FBS and 25 mM HEPES. Cells were then incubated for 45 min at 4°C in this medium with (M + Ab) or without (M – Ab) 16637 rabbit anti-Tfr2 serum, washed twice with PBS, and lysed for 15 min at 4°C in 250 μ l of NETT. Lysates were collected and pooled with a second 250- μ l NETT wash. After the addition of 50 μ l of Pansorbin, samples were rotated for 45 min at 4°C to preclear M – Ab samples or to immunoprecipitate the plasma membrane fraction of Tfr2 from M + Ab samples. On centrifugation of samples at $13,000 \times g$, pellets from M + Ab samples, containing the plasma membrane fraction of Tfr2, were resuspended in 50 μ l of 2X Laemmli sample buffer. To immunoprecipitate intracellular and total fractions of Tfr2, supernatants from M + Ab and M – Ab samples, respectively, were incubated with rabbit anti-Tfr2 serum for 45 min and with 50 μ l of Pansorbin for an additional 45 min at 4°C, centrifuged, aspirated, and resuspended in 2X Laemmli sample buffer. Samples were heated at 95°C for 5 min, centrifuged, and analyzed by SDS-PAGE and Western blot. (B) Tfr2 was detected with mouse anti-Tfr2 antibody followed by a fluorescence-labeled secondary antibody (both 1:10,000). (C) The results from three experiments were quantified.

rects Tfr2 from a degradative pathway to a recycling pathway through recycling endosomes.

In addition to assessing the effect of Fe₂Tf on the steady-state localization of Tfr2 to subcellular compartments, we also examined the effect of Fe₂Tf on the steady-state distribution of Tfr2 to the cell surface. Differential immunoprecipitation was used to isolate sequentially surface and internal Tfr2 from HepG2 cells. In untreated cells, $31 \pm 5\%$ of Tfr2 was at the cell surface and $67 \pm 1\%$ of Tfr2 was intracellular (Figure 3C, open bars). In Fe₂Tf-treated cells, the amount of Tfr2 in surface and intracellular fractions increased relative to untreated cells (Figure 3B). However, the partitioning of Tfr2 between these fractions remained the same ($31 \pm 3\%$ surface versus $71 \pm 2\%$ intracellular; Figure 3C, closed bars). Thus, Fe₂Tf does not alter the proportion of surface and intracellular Tfr2.

Characterization of Wild-Type Tfr2 in Transfected Hep3B Cells

Hep3B cells, in which Tfr2 protein is not detectable (Figure 4A), were used to express Tfr2 mutants to study the mechanism of Tfr2 regulation. Because Tfr2 forms dimers (Kawabata *et al.*, 1999), a null background was particularly important. We first established that regulation of transfected wild-type Tfr2 was similar to that of endogenous Tfr2 in HepG2 cells. Hep3B cells stably transfected to express Tfr2/WT (Hep3B/Tfr2WT cells) regulated Tfr2 in response to Fe₂Tf (Figure 4A). In cells treated with Fe₂Tf, an increased level of Tfr2 protein correlated with an increased half-life of the protein (Figure 4B). The magnitude of stabilization in Hep3B/Tfr2WT cells, from 10 to 28 h, matched that in HepG2 cells, from 4 to 14 h (Johnson and Enns, 2004). Due to the low level of endogenous Tfr2 expression in HepG2 cells, assessment of the effect of Fe₂Tf on Tfr2 biosynthetic rate was not feasible. In Hep3B/Tfr2WT cells, Tfr2 was synthesized at the same rate in untreated and Fe₂Tf-treated cells (Figure 4C), indicating that Fe₂Tf affects the rate of Tfr2 degradation and not its rate of biosynthesis.

We determined the distribution of Tfr2 in Hep3B/Tfr2WT cells by using differential immunoprecipitation to isolate plasma membrane and intracellular fractions of Tfr2. At steady state, $\sim 50\%$ (48 ± 3) of Tfr2 localized to the cell surface and $\sim 50\%$ (48 ± 4) to intracellular compartments (Figure 4D). This distribution was different from that determined for HepG2 cells, in which Tfr2 localized $\sim 30\%$ (31 ± 5) to the cell surface and $\sim 70\%$ (67 ± 1) to intracellular compartments, and the difference is likely a consequence of the high levels of Tfr2 expression in the Hep3B/Tfr2WT cells. Results from uptake experiments using iodinated anti-Tfr2 antibody indicated that the Tfr2 endocytic pathway is saturated in Hep3B/Tfr2WT cells (data not shown), resulting in an accumulation of receptors on the cell surface, as previously seen with overexpression of other receptors (Marks *et al.*, 1996; Warren *et al.*, 1997, 1998).

Confocal microscopy was used to determine whether Tfr2 showed a similar pattern of localization in Hep3B/Tfr2WT cells as in HepG2 cells. Immunofluorescent labeling of Tfr2 at 4°C before fixation and permeabilization detected Tfr2 at the cell surface (Figures 5A, b and e). Under these conditions, Tfr1 at the cell surface can be detected with an antibody recognizing the extracellular domain (3B82A1; Figure 5A, a) but not with an antibody recognizing the intracellular domain (H68.4; Figure 5A, d), indicating that the plasma membrane is intact. Immunofluorescent detection of Tfr2 at room temperature in fixed and permeabilized cells detected intracellular protein, visible as punctate staining in the perinuclear and peripheral regions of the cell (Figure

Figure 4. Fe_2Tf regulates TfR2/WT in Hep3B cells. (A) Fe_2Tf increases TfR2 protein level in Hep3B/TfR2WT cells. Hep3B cells and Hep3B cells stably expressing wild-type TfR2 (Hep3B/TfR2WT) were seeded at 8.3×10^3 cells/cm² in 12-well plates and cultured for 24 h without or with Fe_2Tf . Lysates were analyzed by SDS-PAGE

5B). TfR2 colocalized with EEA1 in early endosomes (Figure 5B, c), TfR1 in early/recycling endosomes (Figure 5B, f), and Golgin97 in the TGN (Figure 5B, i). Together, these experiments established Hep3B cells as a suitable cell line in which to express and characterize TfR2 mutants. In addition, they corroborate previous results indicating that the mechanism of TfR2 regulation by Fe_2Tf is conserved in hepatocyte-derived cells (Johnson and Enns, 2004).

Binding of TfR2 to Diferric Tf Is Prerequisite for TfR2 Stabilization

Our results indicate that Fe_2Tf redirects TfR2 from a degradative pathway through late endosomes and lysosomes into a pathway through recycling endosomes and thereby stabilizes TfR2. Because HepG2 cells express both TfR1 and TfR2, TfR2 stabilization might be consequent on binding of Fe_2Tf to either receptor. Previous efforts to determine which transferrin receptor mediated TfR2 stabilization, using an antibody that blocks and down-regulates TfR1, yielded ambiguous results (Johnson and Enns, 2004). To answer this question directly, site-directed mutagenesis was used to generate a TfR2 construct with mutation G679A. This mutation, in the extracellular domain of TfR2, eliminated detectable binding of TfR2 to Fe_2Tf (Kawabata *et al.*, 2004). If TfR2 stabilization results from interaction of Fe_2Tf with TfR2, rather than from interaction of Fe_2Tf with TfR1, the G679A mutation in TfR2 should eliminate Fe_2Tf -induced stabilization of TfR2. The failure of TfR2/G679A to increase in Hep3B cells transiently transfected with TfR2/G679A and treated with Fe_2Tf (Figure 7, B and C) provided preliminary evidence that interaction of Fe_2Tf with TfR2 stabilizes TfR2.

We therefore went on to characterize the effect of mutation G679A on TfR2 localization and regulation in Hep3B cells stably transfected with plasmid encoding TfR2/G679A (Hep3B/TfR2G679A cells). Confocal microscopy analysis of

and Western blot. Blots were probed with rabbit anti-TfR2 serum (1:10,000) and HRP-conjugated goat anti-rabbit secondary (1:10,000). Protein was visualized by chemiluminescence. (B) Fe_2Tf stabilizes TfR2 protein in Hep3B/TfR2WT cells. Hep3B/TfR2WT cells were seeded at 1.25×10^5 cells/cm² in 35-mm dishes 2 d before the experiment in the absence or presence of 25 μ M Fe_2Tf . Cells were washed twice with PBS and incubated in labeling medium containing 100 μ M [³⁵S]cysteine/methionine without or with 25 μ M Fe_2Tf for 45 min at 37°C. Cells were washed three times with PBS and chased for 2–24 h in normal media in the absence or presence of 25 μ M Fe_2Tf . Lysates were collected, stored at -80°C until all time points were collected, and immunoprecipitated as described in *Materials and Methods*. The stabilization of TfR2 by Fe_2Tf is evident from the autoradiogram (top). Means of measurements from two independent experiments are shown in the graph (bottom). (C) Fe_2Tf does not alter the biosynthetic rate of TfR2 in Hep3B/TfR2 cells. Hep3B/TfR2 cells were seeded at 1.25×10^5 cells/cm² in 35-mm dishes in the absence or presence of 25 μ M Fe_2Tf 2 d before the experiment. Cells were washed twice with PBS and incubated in labeling medium without or with 25 μ M Fe_2Tf for 10–50 min at 37°C. Lysates were collected and immunoprecipitated as described in *Materials and Methods*. Autoradiogram (top) is representative of two independent experiments, the means of which are shown in the graph (bottom). (D) TfR2 localizes to the plasma membrane and intracellular compartments. Hep3B/TfR2WT cells were seeded at 2.5×10^4 cells/cm² in 24-well plates and cultured for 2 d. Differential immunoprecipitation to isolate surface and intracellular fractions of TfR2 was performed as described in Figure 3B. A representative Western blot probed with mouse anti-TfR2 antibody followed by a fluorescence-labeled secondary antibody (both 1:10,000) is shown (top). The averaged results of duplicates from three independent experiments are shown in the graph (bottom).

Figure 5. Subcellular localization of Tfr2/WT in Hep3B cells. (A and B) Hep3B/Tfr2WT cells were seeded at 2.5×10^3 cells/cm² on poly-L-lysine-coated glass coverslips for 24 h before labeling. (A) To detect Tfr1 and Tfr2 at the cell surface, coverslips were incubated at 4°C for 45 min in primary antibodies diluted into ice-cold MEM supplemented with 10% FBS and 25 mM HEPES, pH 7.2, and rinsed twice on ice in ice-cold HBSS before fixation, permeabilization, and labeling with secondary antibodies as described in *Materials and Methods*. Tfr2 was detected with 16637 rabbit anti-Tfr2 serum and is shown in green (b and e). Tfr1 was detected with 3B82A1 mouse antibody recognizing the extracellular domain (ECD) of Tfr1 (a) or with the H68.4 mouse antibody recognizing the intracellular domain (ICD) of Tfr1 (d) and is shown in magenta. The merged images (c and f) show nuclei in blue. The absence of Tfr1 staining in d confirms that intracellular protein was inaccessible to primary antibody. Under conditions that prohibit labeling of intracellular protein, Tfr1 and Tfr2 are visible at the surface of Hep3B/Tfr2WT cells (a–c). (B) Tfr2 colocalizes with EEA1, Tfr1, and Golgin97 in Hep3B/Tfr2 WT cells. To detect total protein by immunofluorescence, cells were fixed, permeabilized, and labeled as described in *Materials and Methods*. Indicated markers are shown in magenta (a, d, and g), Tfr2 is shown in green (b, e, and h), and colocalization is shown in white (c, f, and i). Nuclei are shown in blue in the merged images. Bar, 10 μ m.

cells labeled with anti-Tfr2 antibody at 4°C showed Tfr2/G679A at the cell surface (Figure 6A, b), indicating that this mutation does not prevent transit of Tfr2 through the biosynthetic pathway. Immunofluorescent detection of Tfr2/G679A showed that it colocalized with EEA1 (Figure 6B, c), Tfr1 (Figure 6B, f), and Golgin97 (Figure 6B, i). When Tfr2/G679A was isolated by differential immunoprecipitation, ~30% (33 ± 7) was found in the plasma membrane fraction and ~60% (58 ± 6) was found in the intracellular fraction, a distribution similar to that of endogenous Tfr2 in HepG2 cells (Figure 6C), indicating that this mutation does not affect surface and intracellular steady-state levels of Tfr2.

Given that Tfr2/G679A neither binds nor responds to Fe₂Tf, we predicted that the half-life of Tfr2/G679A would be short and unaffected by Fe₂Tf treatment. In metabolic labeling experiments, Tfr2/G679A had a very short half-life of 2.6 h (Figure 6D), which is significantly shorter than that of endogenous Tfr2 in HepG2 cells and Tfr2/WT transfected into Hep3B cells. Fe₂Tf did not significantly increase this half-life (Figure 6D). From these results, we propose that stabilization of Tfr2 by Fe₂Tf requires direct ligand–receptor interaction.

Preliminary Characterization of Mutations in the Cytoplasmic Domain of Tfr2

Stabilization of Tfr2 by Fe₂Tf involves a change in the trafficking of Tfr2. Because the cytoplasmic domain of mem-

brane proteins often contain signals that direct the protein's trafficking, we used site-directed mutagenesis to alter residues in the cytoplasmic domain of Tfr2 (Figure 7A). V22I is naturally occurring mutation that was speculated to perturb iron homeostasis (Biasiotto *et al.*, 2003). It is adjacent to a putative endocytic motif, YQRV. The YQRV motif is similar to the established endocytic motif in Tfr1, YTRF, in which mutation of the tyrosine decreases the rate of Tfr1 endocytosis (Alvarez *et al.*, 1990; Jing *et al.*, 1990; McGraw and Maxfield, 1990). We generated the corresponding mutation, Y23A, in Tfr2 to assess the role of the YQRV motif in Tfr2 trafficking. K31 is the only lysine residue within the cytoplasmic domain of Tfr2 and is a potential site for ubiquitination, a posttranslational modification that regulates the trafficking and degradation of membrane proteins. We introduced the mutation K31A to assess whether ubiquitination plays a role in the regulation of Tfr2 stability by Fe₂Tf.

To assess whether these mutations affected the ability of Fe₂Tf to regulate Tfr2, Hep3B cells were transiently transfected with constructs encoding the wild-type and mutant proteins. To control for variations in transfection efficiency, a single transfection was split and reseeded before treatment of cells in triplicate without or with Fe₂Tf for 24 h. Western blot analysis using fluorescence-labeled secondary antibodies showed that Tfr2/WT, Tfr2/V22I, and Tfr2/K31A increased significantly in cells treated with Fe₂Tf (Figure 7, B and C). By contrast, Tfr2/Y23A did not respond to Fe₂Tf.

Figure 6. Fe₂Tf binding to Tfr2 is prerequisite for Tfr2 stabilization. (A and B) Hep3B/Tfr2G679A cells were seeded at 2.5×10^3 cells/cm² on poly-L-lysine-coated glass coverslips for 24 h before labeling. (A) Surface Tfr2/G679A was labeled as described in Figure 5A before fixation, permeabilization, and labeling with secondary antibodies as described in *Materials and Methods*. Tfr2/G679A was detected with 16637 rabbit anti-Tfr2 serum (b). Tfr1 was detected with 3B82A1 mouse antibody recognizing the extracellular domain of Tfr1 (a). Bar, 10 μ m. (B) Tfr2/G679A colocalizes with EEA1, Tfr1, and Golgin97 in Hep3B/Tfr2G679A cells. To detect total protein by immunofluorescence, cells were fixed, permeabilized, and labeled as described in *Materials and Methods*. Indicated endosomal markers are shown in magenta (a, d, and g), Tfr2/G679A is shown in green (b, e, and h). In merged images (c, f, and i) colocalization is shown in white and nuclei in blue. Bar, 10 μ m. (C) Mutation G679A does not alter Tfr2 distribution. Hep3B/Tfr2G679A cells were seeded at 2.5×10^4 cells/cm² in 12-well plates and cultured for 2 d. Differential immunoprecipitation to isolate surface and intracellular fractions of Tfr2 was performed as described in Figure 3B. A representative Western blot probed with mouse anti-Tfr2 antibody followed by a fluorescence-labeled secondary antibody (both 1:10,000) is shown (top). The averaged results of duplicates from three independent experiments are shown in the graph (bottom). (D) Fe₂Tf does not stabilize Tfr2/G679A protein. Hep3B/Tfr2G679A cells were seeded and labeled as described in Figure 4B. Labeled Tfr2/G679A was immunoprecipitated and detected as described in *Materials and Methods*. Representative autoradiogram shows the rapid loss of Tfr2/G679A in untreated and Fe₂Tf-treated cells over the course of 7 h (top). The mean of measurements from two independent experiments are shown in the graph (bottom). The rate of Tfr2/G679A decay does not differ significantly in untreated and Fe₂Tf-treated cells ($p = 0.2108$).

This mutant was selected for further characterization in a stable cell line.

A Tyrosine in the Cytoplasmic Domain of Tfr2 Is Critical for Internalization

The Y23A mutation alters a putative tyrosine-based endocytic motif, YQRV, in the cytoplasmic domain of Tfr2. If YQRV acts as an endocytic motif, this mutation should

inhibit internalization of Tfr2. Such an effect might impede normal trafficking of Tfr2 through its degradative pathway and render Tfr2 insensitive to Fe₂Tf. Thus, Tfr2/Y23A should have a long half-life in the absence and presence of Fe₂Tf. Metabolic labeling experiments in Hep3B cells stably expressing Tfr2/Y23A (Hep3B/Tfr2Y23A cells) confirmed this. In marked contrast to both Tfr2/WT and Tfr2/G679A, Tfr2/Y23A was extremely stable, changing little over a 24-h

Figure 7. Response of Tfr2 mutants to Fe₂Tf in Hep3B cells. (A) Sequence of Tfr2's intracellular domain. Residues mutated by site-directed mutagenesis are in bold. The putative endocytic motif is underlined. The position of an additional mutation at G679 in the extracellular domain of Tfr2 is not shown. (B and C) To test the effect of mutations on the response of Tfr2 to Fe₂Tf, Hep3B cells were transiently transfected with constructs encoding wild-type and mutant Tfr2 proteins, replated, and treated in triplicate without (C) or with (T) 25 μ M Fe₂Tf. Lysates were subjected to SDS-PAGE and Western blot analysis. Blots were probed with rabbit anti-Tfr2 serum and mouse anti- β -actin antibody followed by fluorescence-labeled secondary antibodies (all 1:10,000). (B) Representative blots show that Tfr2/WT, Tfr2/V22I, and Tfr2/K31A increase in Fe₂Tf-treated cells, whereas Tfr2/Y23A and Tfr2/G679A do not. (C) Graph shows the averaged results of triplicate treatments from two independent experiments. Significance was assessed with two-tailed Student's *t* test. *p* values of 0.01–0.001 and <0.001 indicate changes that are very significant (**) and extremely significant (***), respectively.

time course, in both the absence and presence of Fe₂Tf (Figure 8A).

To test whether endocytosis of Tfr2/Y23A is impaired, we measured the distribution of Tfr2/Y23A in plasma membrane and intracellular fractions isolated by differential immunoprecipitation. In Hep3B/Tfr2Y23A cells, only ~20% (19 ± 2) of Tfr2 was intracellular (Figure 8B), compared with ~50% in Hep3B/Tfr2WT cells (Figure 4D). Consistent with this observation, no measurable internalization of Tfr2/Y23A was detected compared with the wild-type Tfr2 and Tfr2/G679A, which does not bind Tf (Figure 8C). The altered localization of Tfr2/Y23A was demonstrated by confocal microscopy images of Hep3B/Tfr2Y23A cells that were fixed, permeabilized, and labeled for total protein. Tfr2/Y23A seems blanketed across the cell surface (Figure 8D, b) rather than punctate in the cytoplasm (Tfr1 in Figure 8D, a and Tfr2/WT in Figure 5B, e). Whereas immunofluorescent labeling of Tfr2 at 4°C detected cell surface Tfr2 similarly in both Hep3B/Tfr2WT and Hep3B/Tfr2Y23A cells (Figures 5A, e and 8E, b), immunofluorescent labeling of total Tfr2 showed a cell surface pattern of staining for Tfr2 only in the Hep3B/Tfr2Y23A cells (Figure 8D, b). The results of these experiments are consistent with YQRV as an endocytic motif that mediates Tfr2 internalization from the plasma membrane.

DISCUSSION

In this study, we show that the regulation of Tfr2 by its ligand is different from that of other membrane receptors. Ligand binding to growth factor receptors and G protein-coupled receptors triggers receptor endocytosis and degradation, thereby reducing the amount of surface and intracellular receptor (for review, see Sorkin and Von Zastrow, 2002). The binding of Fe₂Tf to Tfr2, by contrast, increases the amounts of surface and intracellular Tfr2 by inhibiting receptor degradation. The amounts of surface and intracellular Tfr2 increase proportionately. This indicates that the steady state distribution of Tfr2 is unchanged and suggests that ligand binding neither stimulates nor inhibits endocytosis. An analysis of the kinetics of Tfr2 trafficking is required to confirm this.

Fe₂Tf plays a direct role in the stabilization of Tfr2. In transfected Hep3B cells, Fe₂Tf stabilizes Tfr2/WT but does not stabilize Tfr2/G679A, a mutated Tfr2 that does not bind Fe₂Tf (Kawabata *et al.*, 2004). Notably, the half-life of Tfr2/G679A is shorter than that of Tfr2 endogenously expressed in HepG2 cells (Johnson and Enns, 2004) and of

Tfr2/WT stably overexpressed in Hep3B/Tfr2WT cells. This is consistent with the finding that Tfr2, unlike Tfr1, binds appreciably to bovine Fe₂Tf, which is present in tissue culture serum (Kawabata *et al.*, 2004). Medium supplemented with 10% FBS contains ~2.5 μ M (0.2 mg/ml) bovine Tf (Kakuta *et al.*, 1997), a variable fraction of which is fully saturated with iron and capable of binding to Tfr2. Thus, under standard cell culture conditions, Tfr2 levels reflect a basal stabilization by bovine Fe₂Tf.

Mono-ubiquitination of membrane proteins at cytoplasmic lysine residues targets them to lysosomes for degradation. We had hypothesized that mono-ubiquitination of Tfr2 at lysine 31, the only lysine in the intracellular domain of Tfr2, might target Tfr2 for degradation. Thus, mutating the lysine to alanine would stabilize Tfr2, and Tfr2 would no longer be regulated by Fe₂Tf. However, the mutation K31A did not affect regulation of Tfr2 by Fe₂Tf. Our preliminary characterization of the K31A mutation does not exclude the possibility that ubiquitination of lysine 31 might regulate Tfr2 in other ways.

We have identified a residue within the cytoplasmic domain of Tfr2 that is critical for Tfr2 trafficking. Mutation of tyrosine 23 to alanine resulted in a redistribution of the receptor to the cell surface in Hep3B/Tfr2Y23A cells. Tyrosine 23 is part of a tyrosine-based, putative endocytic motif, YQRV, at residues 23–26. Tyrosine-based motifs patterned as YXX Φ , where Φ represents a hydrophobic amino acid, function as sorting signals in the intracellular domains of membrane proteins (for review, see Mellman and Simons, 1992; Marks *et al.*, 1997; Bonifacino and Traub, 2003). The tyrosine residue is required to mediate interaction with the medium (μ) subunit of AP complexes. APs interact with clathrin and thereby concentrate YXX Φ -containing proteins in clathrin-coated pits for vesicular transport within the cell. Our results indicate that tyrosine 23 mediates internalization of Tfr2. This is likely to involve interaction of Tfr2 with AP-2, which functions at the plasma membrane. In Tfr1, a cargo-specific adaptor protein called Tfr trafficking protein (TTP) is also critical for endocytosis (Tosoni *et al.*, 2005). TTP binds to Tfr1 and the endocytic machinery. Whether such an adaptor protein might also specifically mediate Tfr2 endocytosis is not known. AP-1 and AP-3 interact with a subset of YXX Φ motifs to mediate vesicle transport between endosomes and the TGN and to lysosomes and lysosomal-like compartments, respectively. Whether the YQRV motif has additional roles in directing Tfr2 trafficking remains to be determined.

Figure 8. A tyrosine in the cytoplasmic domain of Tfr2 is critical for internalization. (A) Tfr2/Y23A is stable in the presence and absence of Fe_2Tf . Hep3B/Tfr2Y23A cells were seeded and labeled as described in Figure 4B. Labeled Tfr2/Y23A was immunoprecipitated and detected as described in *Materials and Methods*. Representative autoradiogram shows only a slight decrease in Tfr2/Y23A level over the course of a 24-h pulse in both untreated and Fe_2Tf -treated cells. The mean of measurements from two independent experiments are shown in the graph (bottom). The half-life of Tfr2/Y23A in both conditions is >60 h. (B) Mutation Y23A alters Tfr2 distribution. Hep3B/Tfr2Y23A cells were seeded at 2.5×10^4 cells/cm² in 24-well plates and cultured for 2 d. Differential immunoprecipitation to isolate surface and intracellular fractions of Tfr2 was performed as described in Figure 3B. A representative Western blot probed with mouse anti-Tfr2 antibody and a fluorescence-labeled secondary antibody (both 1:10,000) is shown (top). The averaged results of duplicates from three independent experiments are shown in the graph (bottom). (C) The rate of Tfr2/Y23A endocytosis is much less than wild-type Tfr2 and Tfr2/G679A that does not bind Tf. Rates of internalization were analyzed by measuring the rate of disappearance of the receptor from the cell surface by flow cytometry as described in *Materials and Methods*. The results shown are the average of three separate transfections of each construct. (D) Immunofluorescent labeling of cell surface Tfr2 is conspicuous in Hep3B/Tfr2Y23A cells. To detect only cell surface protein, Hep3B/Tfr2Y23A cells were seeded and labeled as described in Figure 5A. Tfr2/Y23A was detected with 16637 rabbit anti-Tfr2 serum (b). Tfr1 was detected with 3B82A1 mouse antibody recognizing the extracellular domain of Tfr1 (a). Bar, 10 μm . (E) Immunofluorescent labeling of total Tfr2/Y23A is similar to that of Tfr2/WT. To detect total protein by immunofluorescence, cells were fixed, permeabilized, and labeled as described in *Materials and Methods*. Tfr1 is shown in magenta (a), Tfr2 is shown in green (b), and colocalization is shown in white (c). Nuclei are visible in blue in the merged image. Bar, 10 μm .

Even though the endocytic motifs of Tfr1 and Tfr2 are similar, the trafficking of these receptors differs. We found that the two receptors only partially colocalized. In addition, Tf traffics to late endosomal compartments in HeLa cells transfected with Tfr2 but not in untransfected cells expressing only endogenous Tfr1 (Robb *et al.*, 2004). This implies a possible role for Tfr2 in Tf sequestration distinct from Tfr1. Finally, whereas Fe_2Tf does not affect the trafficking of Tfr1,

which internalizes constitutively and recycles (Watts, 1985), it does alter the trafficking of Tfr2, redirecting it from a degradative pathway to a recycling pathway.

The mechanism by which Fe_2Tf binding to the extracellular domain of Tfr2 regulates receptor stability and trafficking is unclear. Ligand binding to the extracellular domain might reposition Tfr2 medially in the membrane. This could bury or expose sites for protein interaction or posttranscrip-

tional modification. Alternatively, such repositioning could alter the proximity of residues to the membrane, which can in some cases affect their ability to function as targeting signals (Rohrer *et al.*, 1996). Ligand binding might also disrupt or facilitate interaction between the extracellular domain of Tfr2 and a second membrane protein whose intracellular domain is positioned to interact or mediate an interaction with the intracellular domain of Tfr2. In such a way, an extracellular event could trigger an intracellular response.

The consequence of ligand-induced stabilization, by increasing receptor number, could be the augmentation of a constitutive receptor function, be it signaling, delivering ligand, or interacting with other proteins. Regulated in such a manner, modulation of receptor number could relay changes in ligand concentration. In the present case, this would enable changes in transferrin saturation to modulate processes that maintain iron homeostasis. In healthy individuals, the degree to which Tf in the circulation is saturated with iron generally reflects the supply of iron in the body. Because Tfr2 is expressed in hepatocytes, it is positioned to regulate the expression of hepcidin, a small peptide hormone synthesized and secreted by hepatocytes that controls systemic iron levels by modulating cellular iron efflux (Nicolas *et al.*, 2001, 2002; Pigeon *et al.*, 2001; Roetto *et al.*, 2003; Nemeth *et al.*, 2004). Consistent with this hypothesis, individuals and mice with disease-causing mutations in Tfr2 fail to regulate hepcidin appropriately (Kawabata *et al.*, 2005; Nemeth *et al.*, 2005). Tfr2 might, therefore, sense systemic iron levels through interaction with its ligand.

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