Transferrin Receptor 2-α Supports Cell Growth Both in Iron-chelated Cultured Cells and in Vivo*

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In most cells, transferrin receptor (TfR1)-mediated endocytosis is a major pathway for cellular iron uptake. We recently cloned the human transferrin receptor 2 (TfR2) gene, which encodes a second receptor for transferrin (Kawabata et al. (1999) J. Biol. Chem. 274, 20826–20832). In the present study, the regulation of TfR2 expression and function was investigated. A select Chinese hamster ovary (CHO)-TRVb cell line that does not express either TfR1 or TfR2 was stably transfected with either TfR1 or TfR2-α cDNA. TfR2-α expressing cells had considerably lower affinity for holotransferrin when compared with TfR1-expressing CHO cells. Interestingly, in contrast to TfR1, expression of TfR2 mRNA in K562 cells was not up-regulated by desferrioxamine (DFO), a cell membrane-permeable iron chelator. In MG63 cells, expression of TfR2 mRNA was regulated in the cell cycle with the highest expression in late G1 phase and no expression in G0/G1. DFO was regulated in the cell cycle with the highest expression in late G1 phase and no expression in G0/G1. DFO was regulated in the cell cycle with the highest expression in late G1 phase and no expression in G0/G1. DFO was regulated in the cell cycle with the highest expression in late G1 phase and no expression in G0/G1. DFO was regulated in the cell cycle with the highest expression in late G1 phase and no expression in G0/G1. DFO was regulated in the cell cycle with the highest expression in late G1 phase and no expression in G0/G1.

Iron is essential not only for hemoglobin synthesis in erythroid cells but for a wide variety of cellular functions including electron transport and DNA synthesis. A major pathway for cellular iron uptake is through internalization of the complex of iron-bound transferrin (Tf) and the transferrin receptor (TfR1) (reviewed in Ref. 1). Tf-TfR1 interaction is modulated by HFE, which forms a complex with TfR1 and is defective in hereditary hemochromatosis (2–4). After internalization of the Tf-TfR1-HFE complex, iron is released from Tf as a result of the acidic pH in the endosome. The iron is then transported to the cytosol by DMT1/Nramp2 (5–7). Once in the cytosol, iron is utilized as a cofactor for aconitase, the cytochromes, RNA reductase, and heme (among other proteins), or it is stored as ferritin. After release of iron into the endosome, the resultant apo-Tf recombines with TfR1 in the acidic milieu, and the apo-Tf-TfR1 complex is recruited to the cell surface. On the cell surface, apo-Tf is released in the alkaline pH of the extracellular fluid (reviewed in Ref. 8). Mechanisms for the regulation of expression of TfR1 have been intensively investigated (9–14). TfR1 is up-regulated by iron starvation and down-regulated by iron overload. This regulation is essentially performed at the post-transcriptional level with the binding of iron regulatory proteins to iron-responsive elements on TfR1 mRNA (15). Expression of TfR1 is also regulated at the transcriptional level through the status of cellular proliferation and oxygen saturation (16–18).

Recently we cloned a second receptor for Tf, TfR2 (19). TfR2 codes for at least two alternatively spliced transcripts, α and β. The putative extracellular domain of the TfR2-α protein is highly homologous to TfR1. The TfR2-α protein can bind Tf and mediates iron uptake in CHO cells transfected with TfR2-α cDNA (19). Increased expression of TfR2-α mRNA occurs in the liver (and the hepatoma cell line HepG2) and the erythroid compartment (and K562 erythroid leukemia cells). Thus, cells have at least two distinct receptors for Tf, TfR1 and TfR2-α. Numerous studies have clarified the function, regulation of expression, and clinical relevance of TfR1. In contrast, very little is known about TfR2. In a murine TfR1-knockout model, murine TfR2 could not fully compensate for TfR1 function, and the mice were not able to survive beyond embryonic day 12.5 because of severe anemia and neurologic abnormalities (20). In this study, TfR2 and TfR1 were compared with respect to Tf binding abilities and RNA expression levels. In addition, we have investigated the effects of the expression of TfR2-α protein on cellular growth under in vitro and in vivo conditions.

** EXPERIMENTAL PROCEDURES

Cell Lines—CHO-TRVb (TfR-deficient CHO cell line) and CHO-TRVb-TfR1 (CHO-TRVb cells stably transfected and expressing human TfR1) cells were kindly provided by Dr. T. McGow (21). TRVb-neo (neomycin-resistant control CHO-TRVb cells) and TRVb-TfR2-α (CHO-TRVb cells stably transfected and expressing FLAG-tagged TfR2-α) were established as described previously (19). K562, SK-Hep1 (hepatoma cell line), and MG63 (osteosarcoma cell line) were obtained from American Type Culture Collection (Manassas, VA). K562, SK-Hep1, and MG63 were maintained in RPMI 1640...
medium, Dulbecco's modified Eagle's medium, and minimal essential medium α (Life Technologies, Inc.), respectively, supplemented with 10% fetal bovine serum. CHO-TRVb stable transfectants were maintained in F12 medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum and 200 μg/ml G418. SK-Hep1 cells were transfected with pcDNA3-FLAG-TfR2-α plasmid with Lipofectin (Life Technologies, Inc.). The transfectants were selected with 400 μg/ml G418 and were screened for TfR2-α expression by immunoblotting with the anti-FLAG antibody, M5 (Sigma).

Cell Cycle Synchronization—The cell cycle of MG63 cells was synchronized as described previously (22). Briefly, cells were arrested at G0/G1 by serum starvation for 48 h and then collected either at 24 h after refeeding with medium containing 10% fetal bovine serum and 200 μM mimosine (late G1 phase), 24 h after refeeding with medium containing 2 μg/ml aphidicolin (G1/S phase), 5 h after washing off aphidicolin (S phase), or 30 h after refeeding with medium containing 0.1 μg/ml nocadazole (G2/M phase). Cell cycle synchronization was confirmed by flow cytometric analysis.

Cell Cycle Analysis—The cell cycle was analyzed using the protocol provided by Pharmingen (San Diego, CA). Briefly, cells were pulsed with 10 μM 5-bromo-2-deoxyuridine (BrdUrd) (Sigma) for 1 h, fixed, denatured, neutralized, and stained with fluorescein isothiocyanate-conjugated anti-BrdUrd antibody and propidium iodide, followed by flow cytometric analysis.

Anti-TfR2 Antibody—Polyclonal antibody against TfR2 was developed by Lampire Biological Laboratories (Ottsville, PA) by immunization of a rabbit with glutathione S-transferase-TfR2 (amino acids 514–760) fusion protein. The antiserum was purified with an affinity column (HiTrap NHS-activated, Amersham Pharmacia Biotech) that had been immobilized with the maltose-binding protein-TfR2-β (full-length) fusion construct.

Glycosylation Analysis—FLAG-tagged TfR2-α protein was purified from TfR2-α stably transfected SK-Hep1 cell lysates by immunoprecipitation using the anti-TfR2 antibody. His6-tagged TfR2-α protein was produced in Sf9 cells (insect cell line) using the baculovirus expression vector system (Pharmingen) and was purified using His Bind Resin (Novagen, Madison, WI). The purified proteins were incubated either

![Fig. 1. Deglycosylation of TfR2-α protein.](http://www.jbc.org/)

**A**, histograms of the binding of holo-Tf at several concentrations to cells. CHO-TRVb-neo or CHO-TRVb-TfR1 or CHO-TRVb-TfR2-α cells were incubated with biotinylated Tf followed by incubation with streptavidin-phycoerythrin and analyzed by flow cytometry. Representative results are shown from three identical experiments. **B**, comparison of holo-Tf binding to neo, TfR1, or TfR2-α cells. **C**, apo-Tf binding to neo, TfR1, or TfR2-α cells at increasing pH. **D**, holo-Tf binding to neo, TfR1, or TfR2-α cells at increasing pH. Peak values of the histograms shown in **A** were plotted on **B–D**.
Effect of cellular iron status on expression of Tf receptors in K562 cells.

Cells were cultured with several concentrations of either Fe$_2$(NO$_3$)$_3$ or DFO for 2 days before analysis. A, Northern blot analysis of total RNA (20 μg/lane). The membrane was hybridized sequentially with $TfR1$, $TfR2$, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. B, immunoblot analysis of K562 cell lysates (200 μg) using anti-TfR2 antibody. Cell lysates from TRVb-neo (15 μg) and TRVb-TfR2-α (15 μg) were used as negative and positive controls, respectively. Membrane proteins were visualized by the transillumination method. C, Tf binding analysis. K562 cells were incubated under the designated conditions with 0 (shaded area), 0.1 (solid line), or 3 (broken line) μg/ml biotinylated holo-Tf for 30 min on ice followed by incubation with streptavidin-phycocerythrin. Cell surface binding of holo-Tf was detected by flow cytometry.
with or without PNGase F (New England BioLabs, Beverly, MA) and were detected by immunoblot analysis using either anti-FLAG or purified anti-TfR2 antibodies.

**Flow Cytometric Analysis of Tf Binding**—Tf binding to the cell surface was analyzed by flow cytometry as described previously with some modifications (19). To examine the affinity and capacity of holo-Tf binding to the cells, biotinylated holo-Tf (Sigma) ranging from 0.03 to 100 μg/ml was used. Biotinylated apo-Tf was made by dialyzing biotinylated holo-Tf against 0.1 M sodium acetate, pH 4, overnight. To examine apo-Tf binding at different pH values, cells were incubated

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**Fig. 4.** Effect of cellular iron status on expression of Tf receptors in TRVb-TfR1 and TRVb-TfR2-α cells. Cells were cultured with several concentrations of either Fe₃(NO₃)₃ or DFO for 2 days before analysis. **A,** immunoblot analysis of TRVb-TfR2-α cells using anti-TfR2 antibody. To assess the quality of transferrin, proteins on the membrane were visualized by Ponceau S staining. **B,** cell surface expression of TfR1 on TRVb-neo (shaded area) and TRVb-TfR1 cells. **C,** cell surface expression of TfR2 on TRVb-neo (shaded area) and TRVb-TfR2-α cells. **D** and **E,** cells were incubated with 30 μg/ml biotinylated holo-Tf on ice to achieve maximal Tf binding. Tf binding to TRVb-TfR1 (**D**) and TfR2-α (**E**) cells is shown. **Shaded areas** represent the control experiments in which incubation with biotinylated Tf was omitted. In **B–E,** cells received either no treatment (control, solid lines), 50 μM ferric nitrate (broken lines), or 20 μM DFO (bold lines).
with 5 μg/ml biotinylated apo-Tf in 10 μM desferrioxamine (DFO) (Sigma), 0.1% bovine serum albumin in phosphate-buffered saline on ice for 30 min. DFO was used to avoid converting apo-Tf to ferric Tf during the incubation. In subsequent steps, 0.1% bovine serum albumin in phosphate-buffered saline at the designated pH was used for washes. To analyze holo-Tf binding at different pH values, 0.1% bovine serum albumin in phosphate-buffered saline at pH 7.6 was used for washes to eliminate the binding of apo-Tf.

Analyses for Cell Surface Expression of Tf Receptors—Cell surface expression of TR1 was analyzed by flow cytometry after sequential incubation of the cells with biotinylated CD71 antibody (PharMingen) and streptavidin-phycocerythrin (DAKO, Carpinteria, CA). Cell surface expression of TfR2 was detected by sequential incubation of the cells with anti-TfR2 antibody, biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame, CA), and streptavidin-phycocerythrin.

Clonogenic Assay—Colonies formation in soft agar was examined as described previously with some modifications (23). Approximately 1000 cells were seeded into each well. Different concentrations of DFO and iron loading expression of high affinity Tf receptor (TfR1) decreased, whereas expression of low affinity receptor (TfR2) still remained high.

To examine if TfR2 expression is regulated by iron status at a post-translational level such as during ubiquitination or intracellular trafficking, CHO-TRVb-TfR2-a cells were analyzed for expression levels of TfR2 by Western blot and flow cytometry after treatment with either DFO or ferric nitrate. Immunoblotting using anti-TfR2 antibody showed that the level of TfR2 protein was not affected by a 2-day treatment with either DFO or iron loading. Furthermore, a 2-day exposure to either DFO or ferric nitrate did not change the levels of endogenous TfR2 protein (Fig. 3B). Flow cytometric analysis revealed that exposure of K562 cells to 20 μM DFO markedly enhanced Tf binding to the cells (Fig. 3C, top two panels). In contrast, the affinity of holo-Tf for the iron-loaded K562 cells was lower than that of the untreated control cells (Fig. 3C, bottom three panels). Tf binding to these cells was lower when incubated with 0.1 μM/ml Tf (solid lines) but was almost the same as that of control cells when incubated with 3 μg/ml Tf (broken lines). These results suggest that after iron loading expression of high affinity Tf receptor (TfR1) decreased, whereas expression of low affinity receptor (TfR2) still remained high.

Expression of TfR2 mRNA in MG63 Cells Is Dependent on the Cell Cycle—Northern blot hybridization was performed using human TfR1, TfR2, or β-actin probes. The cell cycle of MG63 cells was synchronized at either G1/S, or G2/M phases. Poly(A)⁺ RNA (2 μg) was loaded onto each lane.

**FIG. 5.** Cell cycle-dependent expression of TfR1 and TfR2 mRNA. Northern blot analysis was performed using human TfR1, TfR2, or β-actin probes. The cell cycle of MG63 cells was synchronized at either G1/S, or G2/M phases. Poly(A)⁺ RNA (2 μg) was loaded onto each lane.

**RESULTS**

**TfR2-a Is a Glycoprotein**—The size of FLAG-tagged TfR2-a protein expressed in CHO and SK-Hep1 cells was approximately 105 kDa, greater than expected from its deduced amino acid sequence. TfR2-a has four putative N-linked glycosylation sites in its extracellular domain suggesting it is a glycoprotein (19). To confirm glycosylation of TfR2-a, we purified FLAG-tagged TfR2-a protein from the lysates of SK-Hep1 cells stably transfected with TfR2-a cDNA. After digestion with N-glycosidase, the size of TfR2-a was reduced from about 105 to 95 kDa (Fig. 1, left). After purification of His₆-tagged TfR2-a protein from Sf9 insect cells, immunoblots showed multiple bands between 95 and 100 kDa. With N-glycosidase digestion, the bands converged at 95 kDa (Fig. 1, right).

Affinity of Holo-Tf for TfR2-a-expressing Cells Is Lower than for TfR1-expressing Cells—A CHO subline that did not express Tf receptors was stably transfected with either neo, TfR1, or TfR2-a. Cells were incubated with several concentrations of biotinylated holo-Tf on ice, and Tf binding to the cell surface was examined by flow cytometry (Fig. 2, A and B). Holo-Tf binding to the neo cells was very low for all tested pH values. If the stoichiometry of apo-Tf was incubated with neo, TfR1, and TfR2-a cells/site in 0.1 ml of Matrixel (Collaborative Biomedical Products, Bedford, MA). Tumor sizes were measured each week and were calculated by the following formula: 0.52 × a × b × c, where a, b, and c are the length, width, and height, respectively.

Other Methods—Northern blot analysis, immunoprecipitation, and immunoblotting were performed as described previously (22). With immunoblotting analysis, the quality of membrane transfer was assessed by either Ponceau-S staining or the transillumination method (from Immobilon-P transfer membrane user guide, Millipore, Bedford, MA). Statistical analysis was done with the Mann-Whitney test.
μg of poly(A)$^+$ RNA from MG63 cells synchronized at G$_0$/G$_1$, late G$_1$, G$_1$/S, and G$_2$/M phases of the cell cycle. Expression of Tfr1 mRNA shows two peaks; maximal expression occurred at G$_1$, and a second peak was observed at the G$_2$/M phase. In contrast, expression of Tfr2 mRNA was not detectable at G$_0$/G$_1$, appeared at late G$_1$, and continued from late G$_1$ through M phase (Fig. 5). We could not detect Tfr2 mRNA at the G$_0$/G$_1$ phase even when 8 μg of poly(A)$^+$ RNA was used in the Northern blot or in reverse transcriptase polymerase chain reaction experiments with 36 cycles of amplification (data not shown).

Cells Stably Transfected with Tfr2-α Are Resistant to DFO—DFO has been reported to inhibit both DNA synthesis and cell proliferation in several cell types (25–27). To examine whether Tfr2-α supports cell growth under conditions of iron deficiency, we performed clonogenic assays using CHO-TRVb stable transfectants (neo, Tfr1, and Tfr2-α) in the presence of DFO. The control neo cells were highly sensitive to DFO, and 5 μM DFO almost completely inhibited colony formation (Fig. 6A). Both Tfr1 and Tfr2-α cells were relatively resistant to DFO, and the number of colonies formed in the presence of 5 μM DFO was only slightly decreased by 15 and 41% in Tfr1- and Tfr2-α-expressing cells, respectively, when compared with clonogenic growth of these cells in the absence of DFO (Fig. 6A). The growth inhibition mediated by DFO was reversed by addition of ferric nitrate to the medium. Cell cycle analysis of CHO-TRVb-neo cells showed that 20 μM DFO reduced the proportion of the cell population in S phase by 71% when determined by incorporation of BrdUrd, and accumulation of the cells in G$_1$/S phase was observed (Fig. 6B, upper panels). In contrast, the proportion of cells in S phase in both the Tfr1 and Tfr2-α cells...
remained high after a 48-h incubation with 20 μM DFO (Fig. 6B, middle and lower panels).

*TfR2-α Promotes in Vivo Growth of CHO-TRVb Cells—*The results from *in vitro* experiments, which indicated growth-supporting effects of TfR1 and TfR2-α prompted us to examine *in vivo* effects of expression of these receptors. CHO-TRVb-neo, CHO-TRVb-TfR1, and CHO-TRVb-TfR2-α cells were inoculated subcutaneously into nude mice, and the size of the resulting tumors was measured. The mean size of TfR2-α tumors was significantly larger than that of neo tumors after 2 weeks. By 5 weeks, all the neo tumors had disappeared, whereas five of the six TRVb-TfR1 tumors and all six of the TRVb-TfR2 tumors had grown. The mean size of the TfR2-α tumors (214.5 mm³) was significantly larger than that of TfR1 tumors (24.9 mm³) at week 4 ($p = 0.013$) (Fig. 7).

**DISCUSSION**

Iron is a cofactor for ribonucleotide reductase, an enzyme that is essential for DNA synthesis (25). If iron is deficient in the cell, both DNA synthesis and cell division are inhibited. DFO retards cell growth through chelation of both intracellular iron. CHO-TRVb cells, lacking functional Tf receptors, were sensitive to iron deprivation by DFO. However, when the cells were stably transfected with either TfR1 or TfR2-α, these cells became more resistant to DFO in analyses of both clonogenic growth and the cell cycle (Fig. 6). The clonal inhibition of growth by DFO was reversed by simultaneous addition of equimolar amounts of ferric nitrate, suggesting that growth inhibition by DFO was caused by iron deprivation (Fig. 6A). These results can be rationalized if TfR2-α as well as TfR1 increases the cellular iron pool and supports cell growth even in the presence of DFO. The animal experiments shown in Fig. 7 indicate that both of these receptors support cell growth in CHO-TRVb cells in nude mice. In contrast, the control CHO-TRVb cells were unable to grow under similar conditions. Expression of the two Tf receptors may enhance cell growth by improving iron availability, which may increase levels of DNA synthesis (Fig. 6B).

In addition to the common effect that TfR1 and TfR2-α have on cell growth, other similarities are evident. Both are glycoproteins (Fig. 1), both have quite similar primary structures, and both can mediate iron uptake through Tf (19). Expression of both TfR1 and TfR2 was cell-cycle dependent in MG63 cells with high expression in late G1 phase, which may be related to the requirement for iron for DNA synthesis during the S phase. Thus, expression of TfR2 as well as TfR1 may be regulated by the cell cycle or cellular proliferation status. In addition, both TfR1 and TfR2-α interact with Tf in a pH-dependent manner; apo-Tf binds to these receptors only at acidic pH and holo-Tf binds at neutral or higher pH. On the other hand, we observed differences between these two receptors with respect to their Tf binding properties and their regulation of expression. A comparison of Tf binding to TfR1 and TfR2-α cells found that the affinity of holo-Tf for TfR2-α was substantially lower than for TfR1. Also, we found that the cellular status of iron had little effect on the regulation of expression of TfR2. DFO enhanced and ferric nitrate reduced expression of TfR1 mRNA in K562 cells (Figs. 3A and 4B), consistent with previous reports (10–13, 24). In contrast, neither DFO nor ferric nitrate affected expression of TfR2 in K562 cells (Fig. 3). This difference between TfR1 and TfR2 may in part be attributed to the fact that TfR2-α mRNA lacks the iron-response element (19). However, if the main function of TfR2-α is to transport iron into the cells, iron overload would be expected to suppress expression of TfR2-α to maintain cellular iron homeostasis. This may suggest another function for TfR2 besides cellular iron uptake. In fact, some of the TfR family proteins such as prostate-specific membrane antigen have peptidase activities (28). The domain that catalyzes this activity (the protease-like domain) is well conserved throughout the TfR family of proteins, though TfR1 has lost this activity (29).

The CHO-TRVb cell system used in this report enabled us to probe the function of TfR2-α compared with TfR1. To evaluate the results from this system, expression of Tf receptors was characterized in the TRVb-TfR1 and TRVb-TfR2-α cells. When cultured in normal medium (5% fetal bovine serum in F12 medium), iron uptake by the TfR2-α cells through Tf was almost the same as that of TfR1 cells (19). Under the same conditions, maximal Tf binding by the TfR2-α cells was 1.7-fold higher than that of the TfR1 cells, which may reflect expression levels of Tf receptors on their cell surface (Fig. 2B). However, expression of TfR1 on the TfR1 cells was clearly up-regulated by preincubation with DFO, whereas expression of TfR2-α on the TfR2-α cells was not affected by the same treatment (Fig. 4). These results, which are similar to those observed in K562 cells (Fig. 3A), can be explained by different cDNA introduced into the cells; the TfR1 cDNA has iron-response elements whereas the TfR2-α gene and cDNA do not. Thus, the expression level of Tf receptors on the TfR1 cells depends on their cellular iron status. Another difference observed by us was that the TfR2-α tumors formed in nude mice were much larger than...
the Tfr1 tumors in vivo (Fig. 7). The cloning efficiency of the Tfr1 cells in the presence of DFO was much greater than that of the Tfr2-a cells (Fig. 6), and this may be related to the increase of Tfr expression mediated by DFO treatment, which occurred in the Tfr1 cells but not in the Tfr2-a cells.

In the majority of individuals with hereditary hemochromatosis, defective Hfe (C282Y) cannot bind to Tfr1. This results in an increase in cellular iron uptake through Tfr (3). However, as the amount of intracellular iron increases, expression of Tfr1 would be expected to decrease as described in previous reports (30, 31) and shown in Fig. 3. This down-regulation of Tfr1 might alleviate cellular iron accumulation. In contrast, expression of Tfr2 did not change significantly in response to iron overload (Fig. 3A). Therefore, in a state of iron overload such as hemochromatosis, the organs which express Tfr2, e.g., liver and erythroid cells, may continue taking iron from serum Fe-Tfr, whereas other tissues may diminish their iron uptake through down-regulation of Tfr1. In evolution, Tfr1 was derived from a peptidase related to prostate-specific membrane antigen (28). Prostate-specific membrane antigen has a higher homology to Tfr2 than to Tfr1, which suggests that Tfr2 may be a more primitive form of Tfr receptor compared with Tfr1. Cells may be controlling iron influx by having two different receptors for Tfr; one is the higher affinity receptor Tfr1 whose expression is regulated by cellular iron status, and the other is the lower affinity receptor Tfr2-a whose expression depends on the cell cycle rather than iron status.

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