The role of transferrin receptor 1 and 2 in transferrin-bound iron uptake in human hepatoma cells

Carly E. Herbison,1,2 Ketil Thorstensen,1,3 Anita C. G. Chua,1,2 Ross M. Graham,1,2 Peter Leedman,1,2,4 John K. Olynky,1,2 and Debbie Trinder1,2

1School of Medicine and Pharmacology, The University of Western Australia, Fremantle Hospital, Fremantle, Western Australia, Australia; 2Western Australian Institute for Medical Research, Nedlands, Western Australia, Australia; 3Department of Medical Biochemistry, The University of Western Australia Centre for Medical Research, Royal Perth Hospital, Perth, Western Australia, Australia; and 4Laboratory of Cancer Medicine, University of Western Australia Centre for Medical Research, Royal Perth Hospital, Perth, Western Australia, Australia

Submitted 19 December 2008; accepted in final form 13 October 2009

The role of transferrin receptor 1 and 2 in transferrin-bound iron uptake in human hepatoma cells. Am J Physiol Cell Physiol 297: C1567–C1575, 2009. First published October 14, 2009; doi:10.1152/ajpcell.00649.2008.—Transferrin receptor (TFR) 1 and 2 are expressed in the liver; TFR1 levels are regulated by cellular iron levels while TFR2 levels are regulated by transferrin saturation. The aims of this study were to 1) determine the relative importance of TFR1 and TFR2 in transferrin-bound iron (TBI) uptake by HuH7 human hepatoma cells and 2) characterize the role of metal-transferrin complexes in the regulation of these receptors. TFR expression was altered by 1) incubation with metal-transferrin (Tf) complexes, 2) TFR1 and TFR2 small interfering RNA knockdown, and 3) transfection with a human TFR2 plasmid. TBI uptake was measured using 59Fe,125I-labeled Tf and mRNA and protein expression by real-time PCR and Western blot analysis, respectively. Fe2Tf, Co2Tf, Mn2Tf increased TFR2 protein expression, indicating that the upregulation was not specifically regulated by iron-transferrin but also other metal-transferrins. In addition, Co2Tf and Mn2Tf upregulated TFR1, reduced ferritin, and increased hypoxia-inducible factor-1α protein expression, suggesting that TFR1 upregulation was due to a combination of iron deficiency and chemical hypoxia. TBI uptake correlated with changes in TFR1 but not TFR2 expression. TFR1 knockdown reduced iron uptake by 80% while TFR2 knockdown did not affect uptake. At 5 μM transferrin, iron uptake was not affected by combined TFR1 and TFR2 knockdown. Transfection with a hTFR2 plasmid increased TFR2 protein expression, causing a 15–20% increase in iron uptake and ferritin levels. This shows for the first time that TFR-mediated TBI uptake is mediated primarily via TFR1 but not TFR2 and that a high-capacity TFR-independent pathway exists in hepatoma cells.

However, there are a number of differences between TFR1 and TFR2. TFR2 has a 30-fold lower affinity for Fe2Tf than TFR1 (25, 44) and, while TFR1 is ubiquitously expressed, TFR2 is predominantly expressed in the liver, suggesting a tissue-specific function. TFR2 protein levels show a dose-dependent response to transferrin saturation, with increasing concentrations of Fe2Tf increase receptor levels by stabilizing TFR2 protein (21, 35). The expression of TFR2 is also regulated by the hemochromatosis protein (HFE) (5). TFR2 is unresponsive to intracellular iron levels (24) unlike TFR1, which is inversely regulated by cellular iron status via the posttranscriptional iron responsive element-iron regulatory protein (IRE-IRP) mechanism. TFR2 cannot compensate for the loss of TFR1 because the TFR1 knockout mouse is embryonic lethal, while TFR1 heterozygote knockout mice display iron deficiency and reduced hepatic iron content compared with wild-type mice (29). Conversely, TFR2 mutations or knockout inactivating the gene in humans and murine models produce an iron overload phenotype with high transferrin saturation and hepatic loading (4, 13, 43). Evidence now suggests that TFR2 plays an important role in the iron-dependent regulation of hepcidin, a peptide synthesized by hepatocytes that coordinates systemic iron homeostasis (12, 23, 33).

The role of TFR2 in hepatic iron uptake is unclear. In liver, TFR1 expression is low and is reduced even further with iron loading, whereas basal liver TFR2 expression is normally high and increases with rising transferrin saturation (21, 35). The high-affinity TFR1-mediated pathway in hepatocytes and HuH7 human hepatoma cells saturates at low transferrin concentrations between 50 and 100 nM in vitro (9, 40). A TFR1-independent uptake pathway for transferrin-bound iron (TBI) has been described in hepatic cells as a low-affinity, higher-capacity process (19, 28, 39, 40). It has been suggested that this process may involve TFR2 (16, 36). However, our recent findings in studies using mouse hepatocytes in vitro are inconsistent with this suggestion, because increased TFR2 protein levels induced by treatment with Fe2Tf were not correlated with an elevation in TBI uptake (18). However, when hepatocytes are isolated from the liver and cultured in vitro, TFR2 expression is reduced to low levels, and small changes in TFR2-mediated TBI uptake may not be detected. Therefore, in the current study we have used an alternative model, a human hepatoma cell line (HuH7) that expresses higher levels of receptors, to determine the relative importance of TFR1 and TFR2 in hepatic TBI uptake and characterized the role of divalent metal transferrin complexes on the regulation of these
receptors. We show for the first time in hepatoma cells that changes in TFR1, but not TFR2 expression, had significant effects on TBI uptake, suggesting that TFR1 is the major TFR responsible for iron transport in hepatoma cells. A range of metal-transferrin complexes can upregulate TFR2 expression but not iron uptake. In addition, there exists a high-capacity pathway for iron uptake which is independent of both TFR1 and TFR2.

MATERIALS AND METHODS

Cell culture. Human HuH7 hepatoma cells were maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Melbourne, VIC, Australia), 2 mM l-glutamine, and gentamicin (80 μg/ml) in an atmosphere of 5% CO2-95% air. Cells were seeded into 6- or 12-well plates and, on the following day, cells were transfected or treated with metal-transferrin complexes. RNA and protein extractions and iron transport experiments were performed 24 h after treatment with metal-transferrin complexes and 48 h after transfection.

Preparation of metal-transferrins. Human Fe2Tf was obtained from Sigma (Castle Hill, NSW, Australia). The transferrin was routinely subjected to size-exclusion chromatography (PD-10 column, GE Healthcare Life Sciences, Rydalmere, NSW, Australia) to remove low-molecular-weight contaminants. Purity of the protein was confirmed by migration of a single band on PAGE, and saturation of transferrin with iron was confirmed by measurement of the absorbance at 470 and 280 nm. Dicobaltic and dimanganic transferrin (Co2Tf and Mn2Tf) were prepared according to the method of Bali and Harris (1). Briefly, CoCl2 or MnCl2 was added to a solution of apotransferrin containing 30 mM NaHCO3 to saturate both binding sites. Hydrogen peroxide was added to oxidize the metal ions to a final concentration of 0.025%, and the solution was incubated at 4°C for 24 h. Excess metal ions were removed by size-exclusion chromatography.

Knockdown of TFR1 and TFR2 by small interfering RNA. HuH7 cells at 30–50% confluency were transfected with small interfering RNA (siRNA; 50 nM/well) using Oligofectamine (Invitrogen) in Opti-MEM serum-free medium (Invitrogen). After 6 h, the transfection medium was replaced with standard incubation medium. A pool of four siRNA oligonucleotides for hTFR2 (siGENOME SMART pool reagent; Dharmacon Research, Lafayette, CO) and a pool of three siRNA oligonucleotides for TFR1 (Stealth Select RNAi, Invitrogen) was used for gene silencing. To control for siRNA treatment, cells were transfected under the same conditions with the Stealth Negative Control LO GC siRNA (Invitrogen). Cells were harvested for analysis at 48 h posttransfection.

TFR2 overexpression. HuH7 cells at 90% confluency were transfected with pcDNA-hTFR2 (26) or pcDNA vector alone (16) with or without TFR2 siRNAs using Lipofectamine 2000 (Invitrogen). The transfection medium was replaced with standard incubation medium after 6 h. Cells were harvested at 48 h posttransfection for analysis.

RNA extraction and RT-PCR. Total RNA was extracted using TRI Reagent (Ambion, Austin, TX) and followed by DNase treatment (DNAfree kit; Ambion). Reverse transcription was performed from 1 μg total RNA using 0.5 μg oligo(dT) primers and 7.5 units AMV reverse transcriptase (Promega, Sydney, NSW, Australia) in a total volume of 10 μl as per the manufacturer’s instructions. TFR1, TFR2, and hpcid mRNA transcripts were quantified by real-time PCR using SYBR green on the Rotor-Gene RG3000 (Corbett Research, Mortlake, NSW, Australia) as described previously (16). The mRNA expression of all genes was normalized against β-actin mRNA expression. Primer sequences used were as follows: β-actin forward 5'-CTGGCACACACCTCTCTA-3', β-actin reverse 5'-GGTTGGTGAAGCTGTAGC-3', hTFR1 forward 5'-AGGAACCGAGTCTCCAGTGA-3', hTFR1 reverse 5'-ATCAACTATGATCACCAGT-3', hTFR2 forward 5'-GGAGGGTGAGGAAGGCTACCTCA-3', hTFR2 reverse 5'-GGTCTTGAGCATAAATCTGTCAT-3' (21), hHepcidin forward 5'-CTGTITTTTCCCCAACACAGCGC-3', and hHepcidin reverse 5'-CAGCACATCCACACTTGA-3'.

Protein isolation and Western blot analysis. After treatment, cells were solubilized in lysis buffer and quantitated as previously described (10). Briefly, 50 μg protein of each sample was incubated in Laemmli buffer and subjected to electrophoresis on 8% [TFR1, TFR2, and hypoxia-inducible factor-1α (HIF-1α)] or 12% (ferritin) SDS-PAGE gels, followed by transfer onto nitrocellulose membrane (PALL Life Sciences). Immunoblot analysis was performed using mouse anti-human TFR1 antibody (1:1,000; Zymed Laboratories, Invitrogen), rabbit anti-human TFR2 antibody [1:5,000 generously supplied by C. Enns, Oregon Health and Science University, Portland, OR (41)], goat anti-human actin antibody (1:800, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human ferritin antibody (1:1,000, DAKO Australia, Kingsgrove, NSW), and mouse anti-

Fig. 1. Expression of transferrin receptor (TFR) 1 and 2 in hepatoma cells treated with metal-transferrins. HuH7 cells were incubated with or without either 30 μM dimeric transferrin (Fe2Tf), dicobaltic transferrin (Co2Tf), dimanganic transferrin (Mn2Tf), or 500 μM desferrioxamine (DFO) for 24 h. TFR1 and TFR2 protein expression was determined by Western blotting. A representative blot is shown in A. The data from 3 independent experiments were quantitated by densitometry, and results are shown in B for TFR2 (dark-shaded bars) and TFR1 (light-shaded bars) following normalization to β-actin, relative to the control treatment (means ± SD; n = 3). C: TFR1 and TFR2 mRNA expression was determined using real-time PCR analysis. Expression was normalized against β-actin and is expressed relative to control. Data represent results from 3 independent experiments (means ± SD; n = 3). *P < 0.05 compared with control value.
human HIF-1α antibody (1:250, Transduction Laboratories, BD Biosciences, San Jose, CA) incubated at 4°C overnight. Secondary goat anti-mouse, goat anti-rabbit, and donkey anti-goat antibodies conjugated to horseradish peroxidase (1:2,000, Santa Cruz) were used to detect immunoreactivity by chemiluminescence (ECL; Amersham Biosciences, GE Healthcare). Images were captured using a VersaDoc Model 3000 (Bio-Rad, Gladesville, NSW, Australia), and protein levels were quantified using Quantity One software (Bio-Rad).

Uptake of iron, cobalt, and transferrin. Human apotransferrin (Sigma) was labeled with 59Fe and 125I (Perkin Elmer, Melbourne, VIC, Australia) as previously described (18, 31). Human apotransferrin was labeled with 57CoCl2 (Perkin Elmer) as detailed above for unlabeled Co2Tf. TFR1-mediated uptake of Fe2Tf was measured at a transferrin concentration of 50 nM, and TFR1-independent uptake (putative TFR2 pathway) was measured at a transferrin concentration of 5 μM, as described previously (9, 28). Uptake of Co2Tf was measured at the same concentrations as Fe2Tf. HuH7 cells were washed twice with Hanks’ balanced salt solution (HBSS), pH 7.4 at 37°C, and then preincubated 2 × 5 min in MEM containing 2% (wt/vol) bovine serum albumin (BSA), pH 7.4 before incubation in MEM-2% BSA containing 59Fe-125I-Tf (50 nM or 5 μM) for 90 min at 37°C. Cells were then washed four times with HBSS at 4°C and incubated for 30 min with Pronase (1 mg/ml in HBSS) at 4°C to distinguish between surface-bound and intracellular uptake of 59Fe and 125I-Tf, as described previously (39). Results are expressed as intracellular uptake, unless otherwise stated. Radioactivity was measured in a LKB-Wallac 1282 compugamma counter (Turku, Finland), and values were normalized against protein concentrations, measured by the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Statistical analysis. RNA and protein results are expressed as means ± SD. Uptake data are expressed as means ± SE. Statistical analysis was performed using unpaired Student’s t-test, and significant differences between groups were accepted at the 95% confidence level.

RESULTS

Effects of metal-transferrin complexes on TFR1 and TFR2 expression and iron uptake. The expression of TFR1 and TFR2 was altered by treating the hepatoma cells with metal-transferrin complexes, Fe2Tf, Co2Tf, or Mn2Tf. This resulted in a 2-fold increase in TFR2 protein levels. Also, Co2Tf and Mn2Tf, but not Fe2Tf, significantly upregulated TFR1 protein levels by approximately twofold. As expected, desferrioxamine (DFO) treatment increased TFR1 expression but did not affect TFR2 protein expression (Fig. 1, A and B). In addition, TFR1 mRNA levels were increased 2.5-fold following treatment with Co2Tf but not with Fe2Tf. There were no changes in TFR2 mRNA levels (Fig. 1C). Similar mRNA results were obtained with Mn2Tf as for Co2Tf (data not shown).

TFR1 is regulated by intracellular iron levels and hypoxia (27, 38). Cobalt is a potent inducer of chemical hypoxia via the stimulation of HIF-1α (22, 30). Therefore, we measured the expression of ferritin, an iron storage protein, and HIF-1α protein to determine whether TFR1 upregulation in the metal-transferrin-treated hepatoma cells was due to a reduction in intracellular iron levels or chemical hypoxia. As shown in
Fig. 2, treatment with Co$_2$Tf, Mn$_2$Tf, or DFO, but not Fe$_2$Tf, increased HIF-1α protein expression. Preincubation with Fe$_2$Tf significantly increased ferritin levels by approximately two-fold. Like DFO, Co$_2$Tf and Mn$_2$Tf decreased ferritin levels by 50%, suggesting that reduced iron levels and hypoxia were responsible for TFR1 upregulation by Co$_2$Tf. Furthermore, treatment of the cells with Co$_2$Tf in combination with ferric ammonium citrate (FAC), a source of non-transferrin-bound iron, reduced TFR1 expression, suggesting that the cellular iron levels were important in the regulation of TFR1 expression (Fig. 2D).

Because the peptide hormone, hepcidin, is affected by both iron status and hypoxia, we examined the effects of Fe$_2$Tf, Co$_2$Tf, and DFO to reduce cellular iron levels, and CoCl$_2$ to induce chemical hypoxia on hepcidin mRNA expression. While hepcidin levels were unchanged with Fe$_2$Tf treatment, Co$_2$Tf and DFO increased hepcidin mRNA expression by 1.5- and 2-fold, respectively. In contrast, CoCl$_2$ reduced hepcidin mRNA levels by 50%, compared with control values (Fig. 2E).

To examine the functional consequences of altered TFR1 and TFR2 expression, we measured TBI uptake by HuH7 cells. As shown in Fig. 3, at 50 nM Tf (TFR1-mediated pathway), iron and transferrin uptake was increased following Co$_2$Tf treatment by 30% and 70%, respectively, reflecting the increase in TFR1 mRNA and protein levels. Fe$_2$Tf treatment, however, did not affect iron and transferrin uptake by HuH7 cells (Fig. 3A and B). At 5 μM transferrin, Fe$_2$Tf treatment did not increase iron and transferrin uptake via the TFR1-independent pathway. However, Co$_2$Tf treatment produced a 40% increase in transferrin uptake without a significant change in iron uptake by the TFR1-independent pathway (Fig. 3C and D). These studies were also carried out in a second hepatoma cell line, HepG2 cells, with similar results (data not shown).

**Uptake of dicobaltic transferrin by hepatoma cells.** To assess whether transferrin can donate cobalt to the cells, HuH7 cells were incubated with 50 nM or 5 μM $^{57}$Co$_2$Tf or $^{59}$Fe$_2$Tf. At each concentration, the amount of cobalt internalized by the cells increased with time, although less efficiently than iron. At 50 nM, the rate of uptake for iron was 4.5-fold higher than that of cobalt (Fig. 4A), while at 5 μM, the rate of iron uptake was 2.5-fold higher than cobalt uptake (Fig. 4B).

**Effect of TFR1 and TFR2 knockdown on transferrin-bound iron uptake.** To further investigate the capacity of TFR1 and TFR2 for TBI transport by HuH7 cells, we performed knockdown experiments using selective siRNAs against TFR1 or TFR2. TFR1 or TFR2 siRNA treatment reduced both their respective receptor mRNA levels by 70% (Fig. 5A), whereas TFR1 and TFR2 protein levels were reduced by 60% and 80%, respectively (Fig. 5B and C). The negative control siRNA had no effect on mRNA or protein expression, and each siRNA pool exhibited no significant cross-reactivity to the other receptor.

At 50 nM transferrin, TFR1 knockdown significantly reduced iron and transferrin internalization by 80% (Fig. 6A and B). The downregulation of TFR1 expression by siRNA had less-pronounced effects on TBI uptake by the TFR1-independent pathway (5 μM Tf) (Fig. 6C). Knockdown of TFR2 alone did not affect iron uptake at either 50 nM or 5 μM transferrin, although a small reduction in transferrin uptake was seen at both concentrations (Fig. 6B and D). The combination of
TFR1 and TFR2 siRNAs had no cumulative effects on TBI uptake and produced results similar to that for TFR1 siRNA alone. At 5 μM transferrin, cells treated with both TFR1 and TFR2 siRNA demonstrated no impairment of iron uptake, indicating that a TFR-independent pathway is involved (Fig. 6, C and D). Knockdown of both TFR1 and TFR2 did not affect hepcidin, hemojuvelin, ferroportin, or divalent metal transporter mRNA expression (data not shown). These experiments were also carried out in HepG2 cells with similar results (data not shown).

**Effect of TFR2 overexpression on transferrin-bound iron uptake.** To maximize the changes in TFR2 protein expression, we transiently overexpressed hTFR2 in HuH7 cells. This upregulation could be attenuated by the addition of TFR2 siRNA (Fig. 7, A and B). TFR1 expression was not altered by TFR2 overexpression, and TFR2 protein expression was much greater than TFR1. Overexpression of TFR2 correlated with a modest 20% increase in the amount of iron internalized. Transferrin internalization, however, increased by twofold. TFR2 siRNA reduced iron and transferrin uptake to control levels, indicating that these effects were specifically due to changes in TFR2 expression (Fig. 8, A and B). Ferritin protein expression consistent with intracellular iron uptake was increased by 15–20% with TFR2 overexpression and was reduced to control levels following TFR2 siRNA treatment (Fig. 8C). Hepcidin, hemojuvelin, ferroportin, and divalent metal transporter1 mRNA expression were not modified by TFR2 overexpression (data not shown).

**DISCUSSION**

This study examined the relative importance of TFR1 and TFR2 in TBI uptake by HuH7 human hepatoma cells and characterized the role of metal-transferrin complexes on the
regulation of these receptors. We showed that changes in TFR1, but not TFR2 expression, had significant effects on TBI uptake at low transferrin concentrations, suggesting that TFR1 is the major TFR responsible for iron transport in hepatoma cells. However, knockdown of both TFR1 and TFR2 had little effect on iron uptake at higher transferrin concentrations, indicating that there exists a high-capacity pathway for iron uptake, which is independent of both TFR1 and TFR2. Treatment of hepatoma cells with metal-transferrin complexes resulted in increased TFR2 protein levels, indicating the importance of the metal binding site occupation rather than the type of metal bound to transferrin in upregulation of TFR2. Fe$_2$Tf stabilizes TFR2 protein by extending its half-life and redirecting it from a degradative pathway to a recycling endosomal pathway (20). It is also possible that Co$_2$Tf and Mn$_2$Tf increased TFR2 protein expression by a similar mechanism. The binding of the dimetal-transferrin complexes to the ectodomain of TFR2 presumably induces an equivalent response from the cytoplasmic domain which has been shown to be largely responsible for its stabilization (6).

Treatment with the iron chelator, DFO, resulted in an upregulation of TFR1 expression which is consistent with previous studies (2, 38). Interestingly, TFR1 mRNA and protein expression were increased at least twofold by preincubation with Co$_2$Tf and Mn$_2$Tf. Our results suggest that the metal ions affect TFR1 expression by two mechanisms. The first is that uptake of transferrin-bound cobalt or manganese, in lieu of iron, results in upregulation of TFR1 expression by activation of the IRE-IRP regulatory mechanism to compensate for the reduction in cellular iron uptake. The depletion in ferritin levels in the Co$_2$Tf- and Mn$_2$Tf-treated cells is consistent with IRP activation. Indeed, Li et al. (30) report IRP1 activation and reduction in ferritin protein, with Co(II) and Mn(II) treatment in A549 cells. In addition, the Co$_2$Tf transport experiments

Fig. 6. Effect of TFR1 and TFR2 knockdown on cellular uptake of iron and transferrin. HuH7 cells were transfected with siRNAs directed against either TFR1 (T1), TFR2 (T2), both TFR1 and TFR2 (T1T2), or negative control siRNA for 48 h. Cellular uptake of iron and transferrin was measured by incubating the cells with 50 nM (A and B) and 5 μM (C and D) $^{59}$Fe-$^{125}$I-transferrin for 90 min at 37°C. Internalized iron (A and C, dark-shaded bars) and transferrin uptake (B and D, light-shaded bars) (means ± SE; n = 3 replicates) are shown. Data are representative of 3 independent experiments. *P < 0.05 compared with control.

Fig. 7. Effect of TFR2 overexpression and knockdown on TFR1 and TFR2 expression. HuH7 cells were transfected with a human TFR2 plasmid in the presence or absence of TFR2 siRNA and assayed for mRNA and protein expression 48 h later. A: mRNA was measured by real-time PCR. Expression was normalized to β-actin and is expressed relative to control cells (means ± SD; n = 3). Similar results were obtained in 2 experiments. *P < 0.01 compared with control, #P < 0.05 compared with TFR2 plasmid. B: protein was quantified by Western blot. This blot is representative of 3 experiments.
suggest that transferrin can donate cobalt to the hepatoma cells, although less efficiently than it does iron, supporting the concept that cobalt and iron are both taken up by the hepatoma cells via TFRs (7). Similar work performed with Mn2Tf indicates that uptake by developing erythrocytes also involves TFR-mediated endocytosis, although the efficiency of uptake and the affinity of the receptors for Mn2Tf was lower than for Fe2Tf (11). This may reflect conformational changes in the transferrin molecule with different metals resulting in altered affinities.

The second mechanism involves regulation via oxygen homeostasis. HIF-1 regulates cellular oxygen homeostasis via transcriptional activation of a number of genes to combat reduced oxygen supply. It is composed of an α- and β-subunit that heterodimerize and bind to hypoxia response elements in the promoter of target genes such as TFR1 (38). HIF-1α remains undetectable in conditions of normoxia because, in the presence of oxygen, the regulatory subunit is hydroxylated by iron-dependent prolyl-hydroxylases and degraded. In conditions of hypoxia this does not occur. Cobalt and manganese salts can induce chemical hypoxia via upregulation of HIF-1α (22, 30), presumably by competing with Fe as a cofactor in this process. Similarly, iron deficiency/chelation can stabilize HIF-1α (2), creating conditions that mimic hypoxia. Low oxygen status can also alter the regulation of IRP binding affinity (8, 17, 37), thereby affecting TFR1 and ferritin expression.

The upregulation of TFR1 by Co2Tf resulted in an increase in the amount of iron and transferrin internalized via the TFR1-mediated pathway. Although Fe2Tf and Co2Tf induced the upregulation of TFR2, there was no significant change in iron uptake by hepatoma cells via the TFR1-independent pathway. Transferrin uptake, however, was increased, suggesting that transferrin uptake and donation of iron to the cell may be uncoupled or that there may be some degree of spill-over in transferrin uptake between the two concentrations of transferrin used to study TFR1-dependent and TFR1-independent pathways. The failure of upregulation of TFR2 by metal-transferrins to increase iron uptake by the hepatoma cells is consistent with a limited role for TFR2 as an iron transporter as suggested in our previous studies in hepatocytes treated with Fe2Tf (9).

Co2Tf increased hepcidin mRNA expression in the hepatoma cells. Co2Tf causes cellular iron deficiency and hypoxia, both of which are known to regulate hepcidin (34). We and others have found that hepcidin was also upregulated by DFO in hepatoma cells in vitro (14), suggesting that iron deficiency/chelation may be important in Co2Tf upregulation of hepcidin. By contrast, hypoxia induced by CoCl2 decreased hepcidin levels, similar to low oxygen levels (3, 34). Therefore, in hepatoma cells, iron deficiency and hypoxia had opposing effects on hepcidin expression, and our results suggest that iron deficiency caused by Co2Tf treatment had a greater effect on hepcidin expression.

siRNAs directed against TFR1 reduced protein levels by 60% and TFR1-mediated uptake of iron and transferrin by 70%, highlighting the efficiency of this pathway. TFR2 siRNA knockdown reduced TFR2 protein expression by 80%, which resulted in a small decrease in transferrin uptake although there was no significant change in iron uptake by either the TFR1-dependent or the TFR1-independent pathways. TFR2 siRNA
did not affect the level of TFR1 mRNA or protein. In keeping with this, combined knockdown of TFR1 and TFR2 showed no further effects on iron and transferrin uptake and was only as effective as TFR1 siRNA alone. Furthermore, overexpression of TFR2 in HuH7 cells resulted in a twofold increase in the amount of transferrin internalized by the cell, but iron uptake and ferritin expression was increased by only 15–20%. These findings demonstrate that TFR2 has the capacity to take up iron in hepatoma cells, as reported previously in TFR2-overexpressing CHO cells (16, 26). Although, our results suggest that TFR2 plays only a minor role in total iron uptake by hepatoma cells. Taken together, these observations make it clear that TFR1 is the dominant TFR involved in iron uptake in hepatoma cells. Furthermore, we showed that cells treated with both TFR1 and TFR2 siRNA did not exhibit impaired iron uptake at a high transferrin concentration, providing support for the existence of a TFR-independent pathway in hepatoma cells which involves the low-affinity endocytosis of transferrin as described previously in hepatocytes (9).

TFR2, together with HFE/TFR1, is thought to act as an iron sensor to modulate the synthesis of hepcidin, the hepatic peptide central to iron homeostasis. The current model of iron regulation by hepcidin proposes that, at high Fe₂Tf concentrations, Fe₂Tf binds to TFR1, resulting in the dissociation of HFE from TFR1. HFE then binds to TFR2, which is stabilized by Fe₂Tf. The HFE-TFR2 interaction promotes hemouvelin/bone morphogenetic protein signaling, causing an increase in hepcidin production (32). Recently, it was shown that HFE association with TFR2 increases the affinity of the receptor for Fe₂Tf, resulting in increased TBI uptake (42) in HFE- and TFR2-overexpressing CHO cells. Whether HFE-TFR2 interaction also occurs in hepatic cells remains to be ascertained.

In this study, we have shown for the first time that TFR2 protein is upregulated by metal-transferrin complexes other than Fe₂Tf. This demonstrates that the occupation of the metal-binding site of transferrin is important in the stabilization and upregulation of TFR2 as opposed to the type of metal bound. However, unlike Fe₂Tf, treatment of hepatoma cells with Co₂Tf and Mn₂Tf also upregulated TFR1 expression due to cellular iron deficiency and chemical hypoxia. Changes in TBI uptake by hepatoma cells were clearly associated with changes in TFR1 expression but not TFR2 expression. In conclusion, uptake of TBI occurs primarily through TFR1, but not TFR2, and there exists a high-capacity pathway, independent of both TFR1 and TFR2, for the uptake of transferrin-bound iron in hepatoma cells.

ACKNOWLEDGMENTS

We gratefully acknowledge Caroline Enns (Oregon Health and Science University, Portland, OR) for providing the TFR2 antibody, Hiroshi Kawabata (Kyoto University, Kyoto, Japan) for the TFR2 cDNA, and Mike Epis (University of Western Australia, Australia) for technical assistance.

GRANTS

This work was supported by a grant from National Health and Medical Research Council of Australia (NHMRC) of Australia (404021) to D. Trinder, J. K. Olynyk, and P. J. Leedman. J. K. Olynyk is the recipient of an NHMRC Practitioner Fellowship (513761) and D. Trinder is the recipient of a Gastroenterological Society of Australia Senior Research Fellowship.

DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


