Failure to Release Iron from Transferrin in a Chinese Hamster Ovary Cell Mutant Pleiotropically Defective in Endocytosis

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ABSTRACT
A Chinese hamster ovary cell mutant defective in the receptor-mediated endocytosis of several unrelated ligands (Robbins, A. R., S. S. Peng, and J. L. Marshall, 1983, J. Cell Biol., 96:1064–1071) failed to accumulate iron provided in the form of diferric transferrin. Analysis of the steps of the transferrin cycle indicated that binding and internalization of transferrin proceeded normally in mutant cells. However, the mutant appeared unable to dissociate iron from transferrin, as evidenced by release of diferric transferrin from the mutant versus apotransferrin from the parent. Uptake of ferric ions from the growth medium was enhanced in the mutant.

A large variety of macromolecules are internalized by the cell after binding to ligand-specific surface receptors (1, 2). Endocytosis of these macromolecules is often followed by separation of ligand from receptor, with the ligand proceeding to the lysosomes, where it is degraded, while the receptor returns to the plasma membrane. It has recently been shown that at least some endocytosed ligands are rapidly delivered to an acidic, prelysosomal vesicle (3–5). This compartment, by virtue of its low pH, may be the site of separation of ligand and receptor. In hepatocytes this acidic, prelysosomal compartment is the site of ligand dissociation from the asialoglycoprotein receptor (6). In baby hamster kidney cells, this compartment is the site whence the nucleocapsid of Semliki Forest virus penetrates into the cytoplasm (7). In addition, this compartment is the most likely site for release of iron from transferrin (TF) (8); unlike most other endocytosed ligands, transferrin does not end up in lysosomes but returns as apotransferrin to the cell surface where it dissociates from the receptor (9–13).

A mutant Chinese hamster ovary (CHO) cell pleiotropically defective in receptor-mediated endocytosis has recently been described (14). The response of this mutant (DTF 1-5-1) to several ligands suggested that the endocytosed ligand never encountered an acidic compartment. In this study we compare the steps of the transferrin cycle in DTF 1-5-1 and parental cells.

MATERIALS AND METHODS

Cells and Cell Culture: The isolation of the CHO cell strains WTB (15) and DTF 1-5-1 (14) has been previously described. Growth medium (16), prepared in the Media Supply Unit of the National Institutes of Health (NIH), contained 5% fetal bovine serum (Gibco Laboratories, Grand Island, NY). All cultures were grown at 34°C under 5% CO₂. Suspension cultures were inoculated at 1–1.4 x 10⁵ cells/ml and were harvested 40–48 h later at densities of 3.5–5.0 cells/ml. Monolayer cultures were inoculated at 4 and 8 x 10⁴ cells per 100-mm dish (WTB and DTF 1-5-1, respectively), the medium was replaced after 2 d, and the cells were used in experiments on day 3 at densities of 6 x 10⁶ cells per 100-mm dish.

Preparation of Ligands; Assays of Binding and Uptake: Preparation of diferric human TF, [⁵⁷Fe]TF, and [⁴⁵⁷I]TF was carried out according to previously published procedures (11). [²⁵FeCl₃] was obtained from Amersham Corp. (Arlington Heights, IL). Cells grown in monolayer or suspension cultures were washed three times with RPMI 1640 (prepared in the Media Supply Unit, NIH) containing 0.1% BSA before incubation with ligand. To separate cells in suspension from unbound ligand, we layered 200 μl of cell pellet was cut off and counted in a Beckman 5500 gamma counter (Beckman Instruments, Inc., Palo Alto, CA). Cells in monolayer were separated from ligand by being washed three times with ice-cold medium, then incubated in 1 ml of medium containing 1% Triton X-100 for 5 min, and scraped from the dish with a rubber policeman. All results presented for binding and uptake of TF have been corrected for nonspecific binding by subtracting the values obtained in parallel samples incubated in the presence of a 100-fold excess of nonradioactive TF. Nonspecific binding represented 10–15% of the total binding.
RESULTS

We compared the accumulation of iron in mutant (DTF 1-5-1) and parent (WTB) cells using human diferric \[^{59}\text{Fe}]\text{Tf}. The level of \(^{59}\text{Fe}\) in WTB cells incubated at 37°C with \[^{59}\text{Fe}]\text{Tf} increased linearly over a period of several hours, while in DTF 1-5-1 the level of \(^{59}\text{Fe}\) reached a steady-state in ~10 min (Fig. 1). After 2.5 h cell-associated \(^{59}\text{Fe}\) in WTB was 3.5 times that measured in the mutant.

To determine at which step in the Tf cycle the mutant is defective, we examined binding, internalization, and release of Tf. As shown in Fig. 2, both WTB and DTF 1-5-1 exhibit a single class of high affinity receptors for human diferric Tf, \(K_d = 9\, \text{nM}\) for WTB and \(K_d = 8.4\, \text{nM}\) for DTF 1-5-1. About 60,000 binding sites per cell were measured on WTB at 4°C; DTF 1-5-1 displayed ~1.5-2 times more binding sites per cell.

In previous studies with human K562 cells incubated in the continued presence of Tf, the level of cell-associated Tf at 37°C was twice that at 4°C (11). This same phenomenon was observed in both mutant and parent CHO cells (Fig. 3); at saturating concentrations of ligand, twice as much Tf was cell-associated at 37°C as at 4°C.

Internalization of Tf was examined by measuring the resistance of cell-associated \(^{125}\text{I}-\text{Tf}\) to protease digestion. Virtually all of the ligand could be released from both WTB and DTF 1-5-1 by Pronase after incubation of the cells with Tf at 4°C, but only 25–30% after incubation at 37°C (Table I). Most of this surface-associated fraction of \(^{125}\text{I}-\text{Tf}\) was transient; when subsequent to incubation at 37°C the cells were washed free of unbound ligand at 4°C (no decrease in total cell-associated ligand occurred on washing) then rewarmed to 37°C for 5 min, all but 5–10% of the cell-associated radioactivity was resistant to Pronase (Table I). Thus both DTF 1-5-1 and WTB cells bind and internalize Tf. Neither mutant nor parent appeared to degrade Tf; even after incubation of the cells with \(^{125}\text{I}-\text{Tf}\) for 4 h at 37°C, no trichloroacetic acid-soluble radioactivity was detected.

The final step of the Tf cycle is the release of the internalized ligand from the cell. To measure release, DTF 1-5-1 and WTB cells were incubated with diferric \(^{125}\text{I}-\text{Tf}\) for 1 h at 37°C, washed free of unbound ligand at 4°C, warmed to 37°C for 5 min to allow internalization of the surface-associated Tf (see above), and then incubated with a large excess of nonradioactive diferric Tf. Addition of excess Tf has been shown to increase the dissociation of \(^{125}\text{I}-\text{Tf}\) from K562 cells that had bound and internalized the labeled ligand (11). Under these conditions the rate of release of Tf from the two cell types was quite similar (Fig. 4).

To determine whether Tf was released intact from the CHO cells, we incubated \(^{125}\text{I}-\text{Tf}\), released from DTF 1-5-1 and WTB
iron-containing transferrin more than 30 times more efficiently than apotransferrin (12). Binding of the $^{125}$I-Tf released from DTF 1-5-1 to the cells was identical to the binding measured with authentic diferric $^{125}$I-Tf; binding of the $^{125}$I-Tf released from WTB cells was about one-tenth thereof (Table II). These results are consistent with release of diferric Tf from the mutant and of apotransferrin from the parent. This was confirmed by immunoprecipitation of $^{125}$I-Tf and $[^{59}$Fe]$^{125}$I-Tf following release from the two cell types. As shown in Table III, the ratio of $^{59}$Fe:$^{125}$I in the Tf released from DTF 1-5-1 was identical to that in the Tf preparations added to the cells. In contrast, Tf released from WTB cells contained only 11% of the $^{59}$Fe present in the starting material. The inability of DTF 1-5-1 to accumulate $^{59}$Fe from diferric transferrin must therefore reflect the lack of dissociation of the iron from the carrier protein within the mutant cell.

To survive, DTF 1-5-1 must utilize some alternate iron source. We compared mutant and parent cells with respect to their abilities to accumulate free ferric ions from the medium.

### Table I

| Source of $^{125}$I-Tf | cpm bound | %
|----------------------|----------|------
| Diferric Tf           | 7,716    |      
| Diferric Tf + 250 μM desferrioxamine | 7,934 |      
| Apo-Tf + 5 μM FeCl₃ | 5,478    |      
| Apo-Tf + 5 μM FeCl₃ + 250 μM desferrioxamine | 408 |      
| Released from WTB cells + 250 μM desferrioxamine | 876 |      
| Released from DTF 1-5-1 cells + 250 μM desferrioxamine | 6,418 |      
| Released from WTB + 5 μM FeCl₃ | 5,126 |      

All Tf used in this table was from a single preparation. Diferric and apotransferrin (apo-Tf) were made as previously described (14) and iron saturation was confirmed by the ODM/OAIC ratio of 0.009 for apo-Tf and 0.079 for diferric Tf. Desferrioxamine (Ciba-Geigy Corp., Greensboro, NC) was made up fresh in water from the mesylate salt. Apo-Tf or apo-Tf plus desferrioxamine were incubated at room temperature for 10 min with 5 μM FeCl₃, pH 7.0, before binding was tested. For all binding studies 100,000 cpm of $^{125}$I-Tf were incubated in 200 μl of RPMI/1% BSA containing 2 x 10⁶ K562 cells at 0°C for 60 min. Cell-associated Tf was determined by pelleting the cells and washing them twice with 1 ml of RPMI/1% BSA. Inclusion of a 200-fold excess of unlabeled diferric Tf in duplicate samples allowed a determination of specific binding. Nonspecific binding to K562 cells was ~5%.

### Table II

**Binding of Tf to K562 Cells**

| Method | Source of $^{125}$I-Tf | cpm bound | %
|--------|----------------------|----------|------
| Method 1 | Diferric Tf | 7,716 |      
| Method 2 | Diferric Tf | 7,934 |      
| Method 1 | Apo-Tf + 5 μM FeCl₃ | 5,478 |      
| Method 2 | Apo-Tf + 5 μM FeCl₃ | 408 |      
| Method 1 | Released from WTB cells + 250 μM desferrioxamine | 876 |      
| Method 2 | Released from DTF 1-5-1 cells + 250 μM desferrioxamine | 6,418 |      
| Method 1 | Released from WTB + 5 μM FeCl₃ | 5,126 |      

Cells grown in suspension were washed, resuspended at 5 x 10⁶ cells per ml, and incubated with $^{125}$I-Tf at 10 μg/ml for 45 min at either 4°C or 37°C. Cells were then washed three times in RPMI at 4°C; a portion of the washed cells that had been incubated with Tf at 37°C was rewarmed to 37°C for 5 min and then chilled to 4°C. Aliquots of each sample were removed for measurement of radioactivity (Before Pronase). The remaining cells were treated with Pronase B (Calbiochem-Behring Corp., American Hoehst Corp., San Diego, CA) at 50 μg/ml for 30 min at 4°C, then washed three times in RPMI/0.1% BSA and resuspended in RPMI; the radioactivity of these samples was determined (After Pronase). No significant loss of cells was observed following treatment with Pronase.

### Table III

**Iron Content of Transferrin Recycled by WTB and DTF 1-5-1**

| Source of $^{59}$Fe/125I | %
|--------------------------|------
| Starting material | 0.0184 | 100 |
| WTB Method 1 | 0.002 | 11 |
| WTB Method 2 | 0.002 | 11 |
| DTF 1-5-1 Method 1 | 0.0178 | 97 |
| DTF 1-5-1 Method 2 | 0.0172 | 93 |

Cells on 100-mm dishes were washed then incubated with either diferric $^{125}$I-Tf or $[^{59}$Fe]$^{125}$I-Tf (each at 10 μg/ml) for 20 min at 37°C. Cells were then washed three times with ice-cold RPMI-0.1% BSA and treated according to the following procedures: Method 1—cells were rewarmed to 37°C for 5 min then chilled to 4°C; Method 2—cells were incubated with 10 μM desferrioxamine in RPMI-20 μM acetate, pH 5.0, for 5 min at 4°C then washed and incubated with 10 μM desferrioxamine in RPMI, pH 7.4, for 5 min at 4°C. After treatment by either method, cells were washed once with RPMI-0.1% BSA and then incubated in the presence of nonradioactive diferric Tf (0.5 mg/ml) for 60 min at 37°C. The medium was collected and aliquots were counted to determine the amounts of $^{59}$Fe and $^{125}$I. $^{59}$Fe was also associated with the cells was recovered in the medium. Additional aliquots were incubated with antitransferrin antibody (17); all of the radioactivity (both $^{59}$Fe and $^{125}$I) present in the medium was immunoprecipitable.
both showed linear uptake of $^{59}$Fe (added as $^{59}$FeCl$_3$) from serum-free medium, with the mutant accumulating two to four times more Fe$^{3+}$ than the parent (Fig. 5). Uptake of Fe$^{3+}$ by both cell types was temperature dependent, with negligible uptake measured at 4°C. The $^{59}$Fe taken up by the CHO cells was not released upon subsequent incubation for several hours at 37°C in the presence or absence of unlabeled FeCl$_3$. To ascertain whether increased ferric ion uptake by the mutant reflected smaller intracellular stores of iron, we preincubated DTF 1-5-1 with FeCl$_3$ for 4 h prior to addition of $^{59}$FeCl$_3$. No decrease in the rate of Fe$^{3+}$ uptake by the mutant was observed.

### DISCUSSION

The CHO cell mutant DTF 1-5-1 has previously been shown to exhibit altered endocytosis of diphtheria toxin, lysosomal enzymes, and the lytic RNA enveloped viruses, Sindbis and vesicular stomatitis virus (14). In this report, we show that this mutant is also defective in endocytosis of transferrin. Although binding, internalization, and subsequent recycling of internalized Tf to the cell surface proceeds normally in DTF 1-5-1, the mutant does not accumulate iron from dextrif Tf; Tf released by the mutant was found to contain the original level of iron. Dextrif Tf was released from the mutant at the same rate as apotransferrin was released from the parent (Fig. 4).

The similarity between release rates in the two cell strains was surprising; in K562 cells at neutral pH, the rate of dissociation of apotransferrin was about 60 times faster than that of dextrif Tf. Thus, release from WTB cells might be expected to be significantly faster than release from DTF 1-5-1. We suggest the following explanations for our findings: First, the $K_d$ measured for human dextrif Tf in CHO cells was about 10 times higher than that in K562 (11, 13); some of this difference may stem from an increased rate of dissociation of dextrif Tf from the CHO cells. Second, the rate of release measured is a combination of the rate of return of internalized Tf to the cell surface in addition to the rate of Tf dissociation. The overall cycling time measured in WTB cells is longer than that for K562 or Hep G-2 cells, since at saturating concentration of dextrif Tf only five to six rounds of delivery of $^{59}$Fe occurred per receptor per hour in CHO cells, compared with 10 or more rounds in the human cell lines (11, 18).

Lack of dissociation of iron from Tf was observed on incubation of normal cells with dextrif Tf in the presence of NH$_4$Cl and monensin (17, 18), agents that dissipate intracellular pH gradients (19). A number of similarities between DTF 1-5-1 and NH$_4$Cl-treated cells have been reported (14), suggesting that the defect in this mutant results from failure to present internalized ligand to an acidic environment. Our results are consistent with this interpretation; moreover, since the Tf cycle appears to bypass lysosomes, the defect in DTF 1-5-1 must involve a prelysosomal acidic compartment. Merion et al. recently demonstrated a defect in ATP-dependent acidification of endosomes isolated from mutant CHO cells phenotypically similar to DTF 1-5-1 (20).

An interesting offshoot of this work is the finding that DTF 1-5-1 may obtain as much iron from transport of ferric ions as is obtained by the parent cells via transferrin. The lower uptake of Fe$^{3+}$ in the parent as compared with the mutant cells suggests that utilization of dextrif transferrin may regulate transport of Fe$^{3+}$. 

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