Functional Expression of the Human Transferrin Receptor cDNA in Chinese Hamster Ovary Cells Deficient in Endogenous Transferrin Receptor

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Abstract. Transferrin (Tf) receptor–variant Chinese hamster ovary cells have been isolated by selection for resistance to two Tf-toxin conjugates. The hybrid toxins contain Tf covalently linked to ricin A chain or a genetically engineered diphtheria toxin fragment. The Tf-receptor–variant (TRV) cells do not have detectable cell-surface Tf receptor; they do not bind fluorescein-Tf or ^{125}I-Tf. TRV cells are at least 100-fold more resistant to the Tf-diphtheria toxin conjugate than are the parent cells. The TRV cells have retained sensitivity to native diphtheria toxin, indicating that the increased resistance to the conjugate is correlated with the loss of Tf binding. The endocytosis of fluorescein-labeled α_2-macroglobulin is normal in TRV cells, demonstrating that the defect does not pleiotropically affect endocytosis.

Since these cells lack endogenous Tf receptor activity, they are ideally suited for studies of the functional expression of normal or altered Tf receptors introduced into the cells by cDNA transfection. One advantage of this system is that Tf binding and uptake can be used to monitor the behavior of the transfected receptor.

A cDNA clone of the human Tf receptor has been transfected into TRV cells. In the stably expressing transfectants, the behavior of the human receptor is very similar to that of the endogenous Chinese hamster ovary cell Tf receptor. Tf binds to cell surface receptors, and is internalized into the para-Golgi region of the cell. Iron is released from Tf, and the apo-Tf and its receptor are recycled back to the cell surface. Thus, the TRV cells can be used to study the behavior of genetically altered Tf receptors in the absence of interfering effects from endogenous receptors.

TRANSFERRIN (Tf)[1] is an iron-carrying serum glycoprotein responsible for Fe^{+3} delivery to vertebrate cells (1). Diferric Tf uptake is mediated by binding to a specific cell surface receptor (20). Once bound, the Tf-receptor complex is internalized, via receptor-mediated endocytosis, into an acidic intracellular compartment (for review see reference 8) where the acidic pH facilitates Fe^{+3} release from transferrin. The Tf receptor (Tf-R) and apo-Tf are recycled back to the cell surface. At physiological extracellular pH apo-Tf is released from the receptor, and the recycled receptor participates in multiple rounds of internalization (4, 6).

The general process of receptor-mediated endocytosis has been morphologically well characterized for a number of ligands in a variety of cell types (for review see reference 7). Little is known about the features of the receptors that serve to signal internalization and intracellular routing. The avail-

ability of cDNA clone of the human Tf-R (17, 26) provides a direct approach to these questions since the cloned gene can be specifically mutated in vitro and transfected into cells. The effect of specific mutations on receptor function can then be characterized. An ideal cell line for such studies would be one that is deficient in Tf-R expression. Tf-R is expressed on most cultured cell lines, presumably reflecting the nutritional requirement for iron (10). Thus, studies of the transfected receptor would necessarily be performed in a background of endogenous Tf-R. The presence of the endogenous receptor would prohibit the use of Tf binding and uptake in the characterization of the transfected receptor, although the expression of the receptor could be monitored using anti–human Tf-R monoclonal antibodies. Mouse L cell transformants expressing the human Tf-R have been isolated using human specific monoclonal antibodies (15, 19).

Tf uptake in Chinese hamster ovary (CHO) cells has been characterized, and the properties are similar to those found in other cell types studied (30). Mutant CHO cells that cannot release iron from Tf are able to transport sufficient iron to support cell growth, apparently by increased uptake of iron salts (14). Since CHO cells have an alternative to Tf-mediated iron uptake, it should be possible to select for cells that

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1. *Abbreviations used in this paper*: DT, diphtheria toxin; f-TF, fluorescein transferrin; med 2, medium consisting of 150 mM NaCl, 20 mM Hepes pH 7.4, 1 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂; Tf, transferrin; Tf-R, transferrin receptor; Tf-RA, transferrin-ricin A chain; TRV, transferrin receptor variant.

are deficient in Tf-R expression. Such cells could then be used for studies of the functioning of the transfected human Tf-R. A further advantage of using CHO cells for these studies is that they have been used for many studies of receptor-mediated endocytosis, including the isolation of mutant cells defective in receptor-mediated endocytosis (12, 24).

A strategy for isolating Tf-R–variant CHO cells is to select for resistance to a Tf-toxin conjugate. In the conjugate, Tf replaces the cell surface–binding domain of the toxin. The specificity of the cytotoxicity of the conjugate is conferred by Tf binding to cells. The rationale behind this approach is that Tf-R–variant cells would not specifically bind the conjugate and would thereby display greater resistance than cells expressing high affinity Tf-R. The efficacy of isolating cells deficient in the expression of cell surface markers by selection with toxin conjugates has been previously established (18, 23). Such an approach, using a Tf-ricin A chain (Tf-RA) conjugate, has been used to isolate a Tf-R–modified human leukemia cell line (22).

In this study we report the isolation of Tf-R–variant CHO cells by selection for resistance to two Tf conjugate toxins: a ricin A chain- and a modified diphtheria toxin (DT)-Tf conjugate. The Tf-R–variant cells do not bind detectable amounts of Tf. A human Tf-R cDNA has been transfected into these cells. The behavior of the human receptor in these cells is very similar to that of the endogenous receptor in wild type CHO cells. ^{59}Fe is efficiently internalized by the transfectants when presented to the cells as diferric Tf, demonstrating that the human receptor is biologically active. These results demonstrate the utility of using the receptor-variant CHO cells for structure-function studies of the human Tf-R.

Materials and Methods

Cells and Cell Culture

WTB Chinese hamster ovary cells were used as the parental cell line (29). Cells were maintained in Ham's nutrient F12 medium (Gibco, Grand Island, NY) supplemented with 5% FCS (referred to as complete medium), or in Ham's F12 containing 500 ng/ml Se_2O_3, 5 µg/ml insulin, and 1 mg/ml BSA fraction V (referred to as serum-free medium) at 37°C in a humidified atmosphere of 5% CO_2 in air. Single cell colony lines were isolated by two rounds of cloning ring colony purification.

Ligands

Human Tf (Sigma Chemical Co., St. Louis, MO) was further purified by Sephacryl S-300 gel filtration. Diferric Tf, fluorescein Tf (f-Tf), and ^{125}I-Tf were prepared as described previously (30). ^{59}FeCl$_3$ was purchased from New England Nuclear (Cambridge, MA). Tf^{59}Fe$_2$ was prepared by the nitrilotriacetic acid method (14).

Synthesis of Conjugate Toxins

Diferric Tf (5 mg) was reacted with N-succinimidyl 3-(2-pyridyldithio) proponate (SPDP; Pharmacia Fine Chemicals, Piscataway, NJ) at a molar ratio of 5:1 (SPDP:Tf) as described (22). Approximately 3–5 SPDP groups were added per transferrin molecule, and assayed as previously described (28).

Immediately before coupling, ricin A chain (E. Y. Laboratories, Inc., San Mateo, CA) and DT fragment MspSA (3) were reduced with 1% β-mercaptoethanol for 10 min at 37°C. The reducing agent was removed by G-25 gel filtration. The reduced protein was collected directly into SPDP-derivatized Tf (5 mg) on ice, at a ratio of 5 mol of toxin per mol of Tf. After 10 min on ice the sample was concentrated, using Macrosolute concentrator B-15 (Amicon Corp., Danvers, MA), to ∼6 mg total protein per ml and incubated

at room temperature overnight. The mixture was then fractioned on a Sephacryl S-300 column equilibrated with PBS. The fractions were assayed for the presence of conjugates by nonreducing PAGE. Fractions containing high molecular mass species (M_r > 120,000) were filter sterilized and stored at 4°C. The protein concentrations in these fractions were estimated by measuring the absorbance at 280 nm.

Toxicity Assay

To assay the cytotoxicity of the conjugates, 1 × 10^5 cells were plated per well (1 cm^2) in 24-well plates 16 h before testing. The cells were then washed three times with medium 2 (med 2: 150 mM NaCl, 20 mM Hepes pH 7.4, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$) and then incubated in serum-free Ham's F12 containing the conjugates for 16–18 h. The cells were then washed three times with med 2 and incubated with leucine-free MEM medium (Gibco) containing 2 µCi ^{3}H-leu/ml. After a 2-h incubation, the incorporation of ^{3}H-leu into TCA-precipitable material was determined.

Selection of Mutants

A confluent 150-cm^2 flask of WTB cells was mutagenized with 240 µg/ml of ethylmethyl sulfonate (Sigma Chemical Co.) in complete F12 for 16 h at 37°C. This treatment results in ∼50% cell killing. The cells were then passaged twice in complete F12 over a 6-d period to allow for fixation of the mutations. Half-confluent 100-mm dishes were then incubated with 5 × 10^{-9} M Tf-RA conjugate in serum-free F12 containing 50 nM monensin for 18 h. The cells were washed and maintained in complete F12 for 48 h. The cells were then treated with 5 × 10^{-9} M Tf-MspSA conjugate in serum-free F12 for 18 h. The cells were split 1:2 and maintained for 24 h in complete F12. The cells were reselected with 5 × 10^{-9} M Tf-MspSA as in the second round of selection.

f-Tf Uptake Assay

Cells grown on coverslip dishes (30) were incubated with f-Tf (typically 20–100 µg/ml) in med 2 containing 2 mg/ml ovalbumin for 20 min at 37°C. Fluorescence experiments were conducted using a Leitz Diavert fluorescence microscope. Video images were obtained using a DAGE/MTI 65 MKII SIT camera.

Transfections

The human transferrin receptor clone pCDTR1 (17) and pSV3Neo (26) (10 µg pCDTR1 and 2 µg pSV3Neo for 5 × 10^6 cells) were introduced into cells by the calcium phosphate coprecipitation technique, with the addition of glycerol shock after 5 h (27). After exposure to DNA for 16 h, the cells were trypsinized and replated at a 1:3 dilution in complete medium containing 400 µg/ml G-418 (Gibco). After 10 d the surviving cells were pooled and analyzed. Transfectant cell lines were carried in complete F12 supplemented with 400 µg/ml G-418.

Immunofluorescence

Cells were fixed with 3.7% formaldehyde (freshly diluted in PBS) for 10 min. The cells were washed three times with med 2 before incubation with 5 µg/ml human specific anti–Tf-R monoclonal antibody B3/25 (Boehringer Mannheim Biochemicals, Indianapolis, IN) in med 2 containing 100 µg/ml saponin and 2 mg/ml ovalbumin for 30 min at 37°C. After three 2-min rinses with med 2, the cells were incubated with fluorescein-labeled goat anti–mouse IgG in the same buffer as the first antibody for 30 min at 37°C. The cells were then washed with med 2 and examined using fluorescence microscopy. For comparison with direct uptake of F-Tf the images were recorded using the SIT camera.

Results

Synthesis of the Transferrin Conjugate Toxins

Two conjugate toxins were synthesized in which Tf replaced the cell surface–binding domain of the toxin. In one case Tf was coupled to ricin A chain and in the other it was coupled to a genetically engineered DT fragment MspSA (3). MspSA is a cloned DT gene in which the COOH-terminal 17,000 D

Table I. Tf-RA Conjugate Inhibition of Protein Synthesis of WTB

Treatment	Protein synthesis (percentage of untreated cells)
Tf-RA $(5 \times 10^{-9}$ M)	122 ± 6
Tf-RA $(5 \times 10^{-9}$ M) + 50 nM monensin	48 ± 5
Tf-RA $(5 \times 10^{-9}$ M) + 50 nM monensin + Tf $(2 \times 10^{-6}$ M)	105 ± 6
50 nM monensin	103 ± 4
Unconjugated RA $(10^{-7}$ M)	43 ± 5

CHO wild type (WTB) cells were incubated with Tf-RA for 16 h and assayed for protein synthesis as described in Materials and Methods. Values are expressed as percent of ^{3}H-leu incorporation in untreated control cells. Values $\pm$SEM, based on three determinations.

of the B chain, containing the cell-surface binding site of DT, have been deleted. After gel filtration, the conjugate-containing fractions were identified by nonreducing PAGE. In both cases two distinct bands migrating at the expected location for conjugate species of one and two toxin fragments per Tf molecule were observed. The intensities of these bands in Coomassie-stained gels indicated that the two conjugate forms were present in approximately equal amounts. The conjugate fractions also contained some unconjugated TF (<20%) and free toxin chains (<10%). No attempt was made to further purify the conjugates because the unconjugated toxin fragments are relatively nontoxic when compared with those conjugated to molecules that provide high affinity cell binding (3, 22).

The two conjugates displayed different activities in an inhibition of cellular protein synthesis assay. The TF-RA conjugate, at a concentration of 5×10^{-9} M, had no effect on protein synthesis when incubated with WTB cells for 16 h (Table I). The toxicity of Tf-RA could be potentiated by incubation in the presence of 50 nM monensin, which has been shown to potentiate the toxicity of ricin and ricin A chain conjugates in other systems (2). When 5×10^{-9} M Tf-RA was incubated with cells in the presence of 50 nM monensin, protein synthesis was reduced to ~50% of the control level (Table I). The toxicity was completely blocked by a 500-fold excess of Tf. Monensin (50 nM) alone had no effect on protein synthesis.

The Tf-MspSA conjugate was a potent inhibitor of protein synthesis, reducing protein synthesis to 50% of control levels at a concentration of 1×10^{-10} M (Fig. 1). A concentration of 2×10^{-7} M unconjugated MspSA reduced protein synthesis to 50% of control level. The effect of the Tf-MspSA was completely blocked by inclusion of a 500-fold excess of Tf during incubation with the conjugate, demonstrating that the specificity of the conjugate is conferred by the Tf binding (Fig. 1).

The most likely explanation for the differences in the effects on protein synthesis is the efficiency with which the A chains of the conjugates are able to gain entry to the cytoplasm. The MspSA fragment contains a portion of the DT B chain believed to be involved in the transport of the A chain into the cytosol (3). Similarly, it has been proposed that portions of the ricin B chain are also required for efficient cyto-

solic entry of the A chain (2). In the Tf-RA these regions of the toxin are not present, and this could explain lower toxicity of the Tf-RA conjugate.

## Selection of Transferrin Receptor-Variant WTB Cells

The conjugates were used to isolate a Tf-R-variant WTB cell line by selection for growth in the presence of the conjugates. Three rounds of selection were used to isolate Tf-R-variant WTB cells (Materials and Methods). After the third round of selection, an aliquot of the surviving cells was tested for uptake of f-TF using fluorescence microscopy. A spectrum of f-TF uptake patterns ranging from no detectable uptake to apparently normal uptake was observed. Approximately 40% of the cells showed no fluorescein fluorescence, indicating that they did not bind or internalize detectable amounts of f-Tf. Pure cell lines were isolated by two rounds of single cell purification. The f-Tf uptake pattern of 11 cell lines was examined and two cell lines, which did not have detectable f-Tf uptake, were chosen for further study. These cell lines have been designated TRVa and TRVb. These cell lines have been continuously passaged in the absence of selection for a period of 6 mo without any perceptible alteration, as assayed by f-Tf uptake, in the receptor-variant phenotype. The mutant cells proliferate in complete Ham's F12 media at a rate similar to the parent WTB cells.

Characterization of Transferrin Receptor-Variant CHO Cell Lines

WTB cells, as do other cell lines (6, 9, 11, 29) juxta-nuclear, sequester f-Tf into a para-Golgi compartment comprised of small vesicles and tubules (Fig. 2 A; 30). f-Tf uptake is a sensitive assay for cell-associated Tf because the fluorescein fluorescence is concentrated into a localized region of the cell. To further maximize the sensitivity of the assay, the uptake studies were performed using 100 µg/ml of f-Tf, which is ~20 times the saturating concentration of human ^{125}I-Tf binding to WTB cells. TRVa cells do not display any cell-
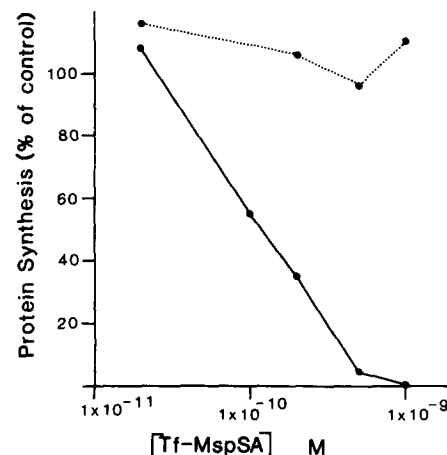
Figure 1. Effect of Tf-MspSA conjugate on WTB cells. WTB cells plated in 24 well plates were incubated for 18 h at 37°C in the presence of Tf-MspSA (———); or in the presence of Tf-MspSA and a 500-fold excess of Tf (- - - -). The samples were then assayed for protein synthesis as described in Materials and Methods. The results are expressed as a percentage of ^{3}H-leu incorporation in untreated control cells.
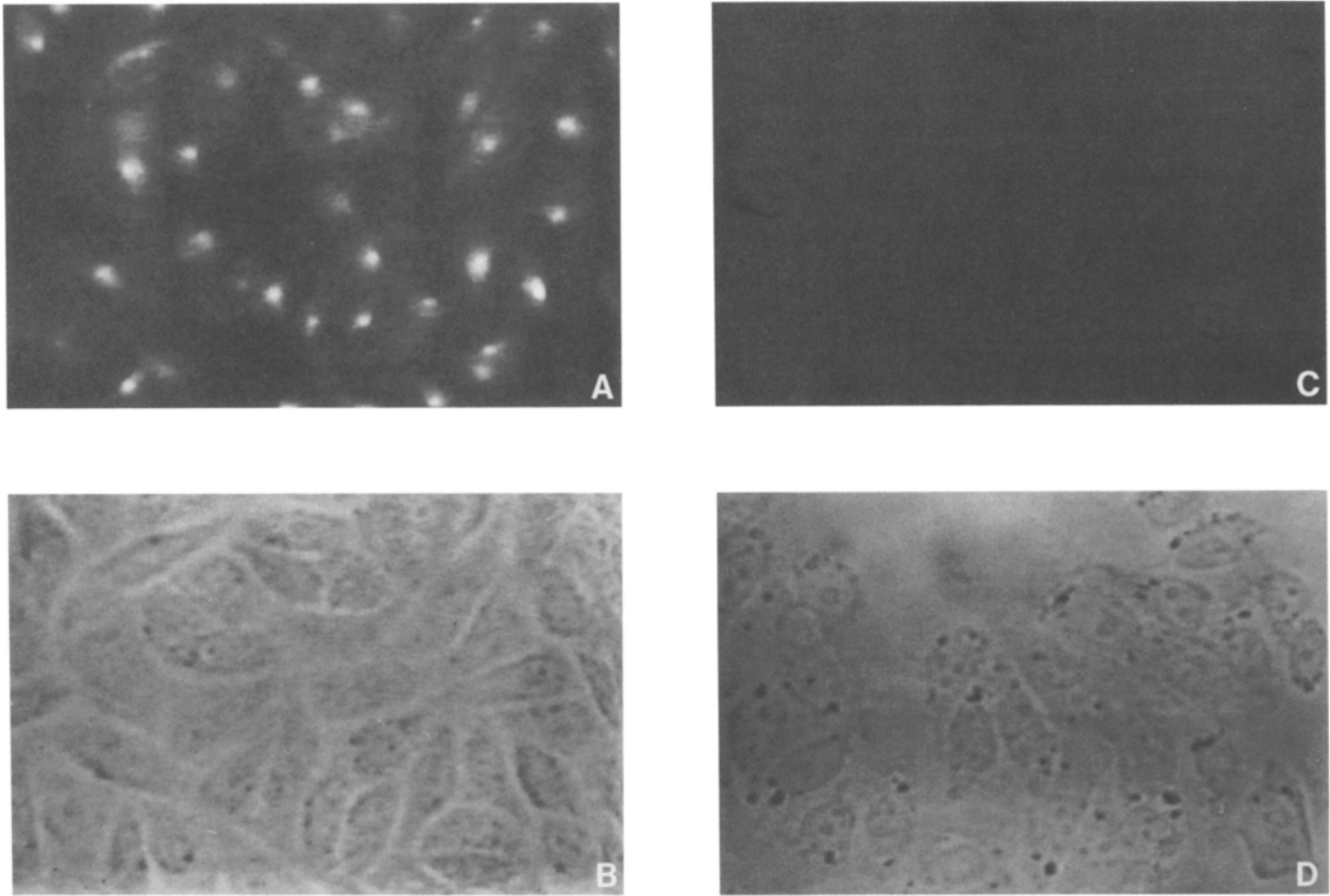
Figure 2. f-Tf uptake in WTB and TRVa mutant cells. Cells were incubated with 100 μg/ml of f-Tf for 20 min at 37°C, rinsed free of ligand, and fixed in 3.7% formaldehyde for 10 min. (*A*) Fluorescein fluorescence of WTB cells. (*B*) Phase contrast illumination of same field in *a*. (*C*) Fluorescein fluorescence of TRVa cells, and (*D*) phase contrast of same field. No f-Tf fluorescence could be detected in the TRVa cells; identical results were obtained with TRVb cells.

associated f-Tf (Fig. 2 *C*). No para-Golgi fluorescence is observed indicating that there is no detectable f-Tf uptake. Furthermore, there is no overall increase in cellular fluorescence suggesting that TRVa cells are defective in f-Tf binding and not in internalization or delivery to the para-Golgi compartment. Identical results were obtained with the other mutant cell line, TRVb (not shown).

A more quantitative assessment of Tf binding to the mutant cells was provided by examining the uptake of ^{125}I-Tf (not shown). No specific uptake was observed when TRVa or TRVb cells were incubated at 37°C for 20 min with 7 μg/ml ^{125}I-Tf (∼15 times half-maximal binding concentration).

The Tf-R–variant phenotype of these cells was correlated with their increased resistance to the Tf-MspSA conjugate. A concentration of 1 × 10^{-9} M Tf-MspSA, which completely inhibits protein synthesis of the parent cell line, had no effect on the mutant cells (Fig. 3). Since Tf-MspSA concentrations above 10^{-11} M reduced protein synthesis of WTB cells (Fig. 1), the mutant cell lines are at least 100-fold more resistant to the conjugate than the parent cell line. The increased resistance is due to a defect in the Tf uptake and not due to an inability of DT to intoxify cells since the mutant cells are as sensitive to complete DT as are WTB cells (Fig. 3).

The serum protease inhibitor, α₂-macroglobulin, is also

internalized by receptor-mediated endocytosis (30). The pattern of fluorescein-labeled α₂-macroglobulin uptake in the mutant cells was indistinguishable from that of parent WTB cells (not shown). This result, along with the sensitivity to DT, confirms that the mutant cells are specifically defective
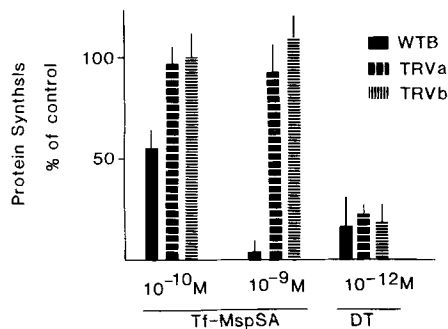
Figure 3. Effect of Tf-MspSA and DT on WTB, TRVa, and TRVb cells. Cells were incubated with the indicated concentration of DT or Tf-MspSA for 18 h and then assayed for protein synthesis as described in Materials and Methods. The mutant cell lines are resistant to Tf-MspSA but not to DT.
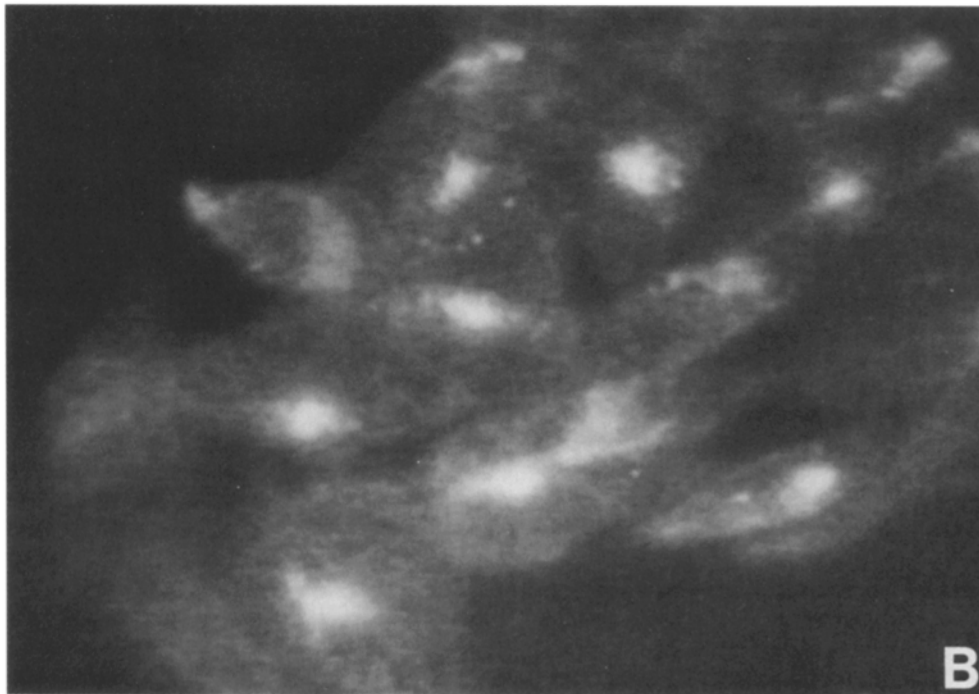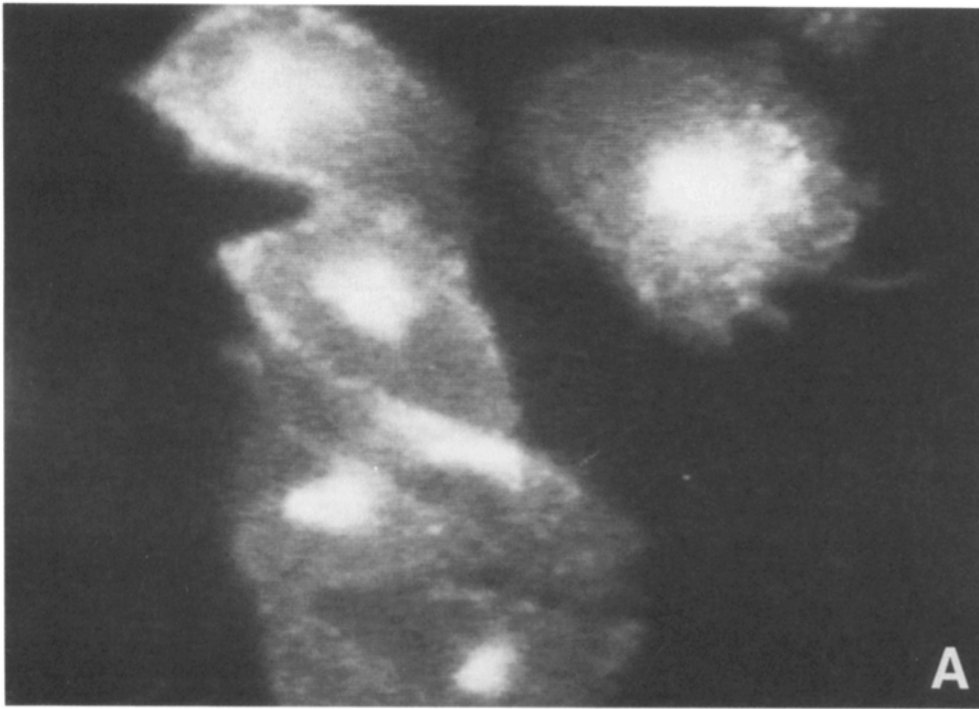
Figure 4. f-Tf uptake and immunofluorescence of TRVb-1 cells. (*A*) f-Tf uptake pattern of TRVb-1 cells incubated with 20 μg/ml f-Tf for 20 min at 37°C. (*B*) Fixed and permeabilized TRVb-1 cells were treated with a monoclonal antibody specific for the human Tf-R, followed by incubation with a fluorescein-labeled goat antimouse antibody. Similar results were found with the TRVa-11 cells. In both cases, the fluorescence was easily observed by conventional fluorescence microscopy. The images shown were recorded using a SIT camera.

in Tf uptake, presumably due to a defect in the Tf-R or its expression, and are not pleiotropically defective in endocytosis or in toxin entry.

Expression of Human Transferrin Receptor in the TRV Cell Lines

The cDNA clone of the human Tf-R was transfected into the mutant cell lines to establish the efficacy of using these cells

for studies of the function of the receptor. The cDNA, pCDTR1, was transfected into both mutant cell lines using Ca–phosphate coprecipitation with the dominant selectable marker plasmid pSV3-Neo. For transfection pCDTR1 was cut with Bam HI, which releases a 5-kb fragment containing the complete cDNA insert plus ~600 bp of flanking vector sequences. This approach was adopted because when uncut pCDTR1 was used in transfection experiments, no expres-
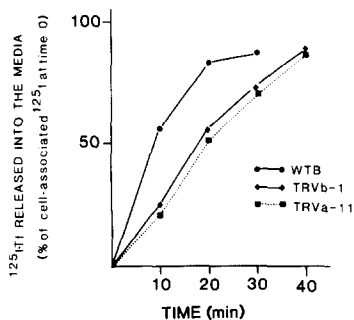
Figure 5. ^{125}I-Tf washout studies of WTB, TRVa-11, and TRVb-1 cells. Confluent monolayer of cells in six well plates were incubated with 5 µg/ml ^{125}I-Tf (540 cpm/ng) for 20 min at 37°C. The cells were washed three times with med 2 to remove unbound ligand. The cells were reincubated in med 2 containing 100 µM of the iron chelator Desferroxamine (Ciba-Geigy Corp., Greensboro, NC) at 37°C. Under these iron-free incubation conditions the recycled apo-Tf does not rebind to cells. At various times after washing, the ^{125}I released onto the medium was determined by gamma counting. Greater than 90% of the released label was TCA precipitable.

sion of the Tf-R was observed (Ruddle, F., personal communication). The reason for the block in expression of the receptor gene in the intact pCDTR1 construct is not known. The G-418 resistant cells were pooled and screened for uptake of f-TF. Approximately 30% of the cells were positive for f-Tf uptake. The majority of Tf uptake positive cells displayed the expected pattern for Tf internalization: concentration of the ligand in the para-Golgi region. Transfection for G-418 resistance alone does not alter the Tf-R–variant phenotype of the mutant cells. Pure TF-R–positive transfectant cell lines from both TRVa and TRVb were isolated (designated TRVa-11 and TRVb-1) and the expression of the human receptor in these cells was characterized.

The f-Tf uptake pattern in the transfectants is indistinguishable from that of WTB cells, with the ligand being rapidly concentrated into the para-Golgi compartment (Fig. 4 *A*). Immunofluorescence of the permeabilized transfectants using a monoclonal antibody to the human Tf-R, B3/25, confirms that it is the human receptor that is expressed in the transfectant cell lines (Fig. 4 *B*). The immunofluorescence
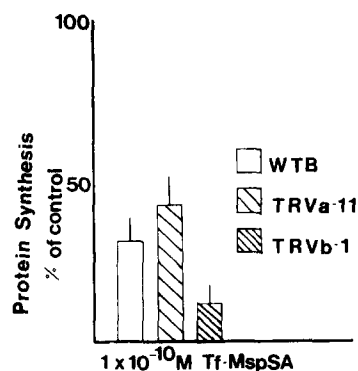
Figure 6. Tf-MspSA effect on TRVa-11 and TRVb-1 cells. The parental cell line and the two mutant lines containing transfected human Tf-R were assayed for sensitivity to Tf-MspSA. Assay conditions were identical to those of Fig. 3. The human Tf-R restores sensitivity to the toxin conjugate.

pattern is identical to the f-Tf uptake pattern demonstrating that the human Tf-R is sorted into the same region of the cell as f-Tf. It is from this compartment, the para-Golgi, that the receptor-ligand complex is presumably returned to the cell surface. (It should be noted that in CHO cells, because of the unavailability of anti–receptor antibody, it has not been directly demonstrated that the receptor is recycled to the cell surface by the same pathway as Tf.) As expected, the human specific monoclonal B3/25 does not stain WTB cells (not shown).

[125]I-Tf binding at 4°C to the transfectant cells was examined and K_ds of 1.6 nM for TRVa-11 and 3.4 nM for TRVb-1 were estimated from Scatchard plots of the data. This value is in accordance with previously reported K_ds for human Tf binding to human cells (13). TRVa-11 cells were found to have 25,000 surface receptors/cell, and TRVb-1 to have 150,000 surface receptors/cell. The disparity in expression of the receptor is a feature of the individual transfectants chosen for this study and not a characteristic of the mutant cell lines. Analysis of other transfectants revealed a similar range of expression of the receptor in both TRVa and TRVb transfectants. Values of K_d = 45 nM and 65,000 surface receptors/cell were found for WTB cells.

Washout studies were performed to establish that f-Tf internalized by the human receptor in the transfectants is recycled. Cells were preloaded with f-Tf by incubation for 20 min at 37°C. The cells were washed to remove unbound f-Tf and reincubated in Tf-free medium containing the iron chelator desferoxamine (100 µM). Desferoxamine was included in the media to bind iron and thereby block the iron reloading of recycled apotransferrin. The f-Tf fluorescence in the para-Golgi of the transfectants was lost over a 40-min period. Similar results were observed in WTB cells. The loss of fluorescence under these conditions is a result of the recycling of apo-Tf, which in the absence of iron does not bind to the receptor at neutral pH. These results indicate that at the level of light microscopy the transfected human receptor binds Tf, is internalized, sorted, and recycled in a fashion similar to the CHO receptor.

The time course of release of ^{125}I-Tf from the cells was studied (Fig. 5). The release of Tf was somewhat slower in the transfectants than in WTB cells. In all cases, >90% of the released ^{125}I-Tf was TCA precipitable, indicating that the ligand had not been degraded.

TRVa and TRVb cells are resistant to the Tf-MspSA conjugate because they are deficient in Tf binding. Therefore transfectant cells should, by virtue of expression of the human receptor, have regained sensitivity to the conjugate. As shown in Fig. 6 the transfectant cells were sensitive to the action of Tf-MspSA. A direct comparison of the sensitivity of the transfectants and the parent cells is not warranted because of the differences in receptor number and affinity for Tf. Regardless, the expression of the human receptor results in a reversion of the Tf-MspSA resistant phenotype.

The ability of the transfectants to accumulate ^{59}Fe from Tf[59]Fe$_2$ supplied in the medium was investigated. WTB cells and both of the transfectants were able to accumulate ^{59}Fe whereas the mutant cells were not (Fig. 7). The ^{59}Fe accumulation in WTB, TRVa-11, and TRVb-1 cells was inhibited by inclusion of an excess of unlabeled Tf in the incubation media. The differences in absolute amounts of ^{59}Fe accumulation reflects the differences in the number of Tf-Rs
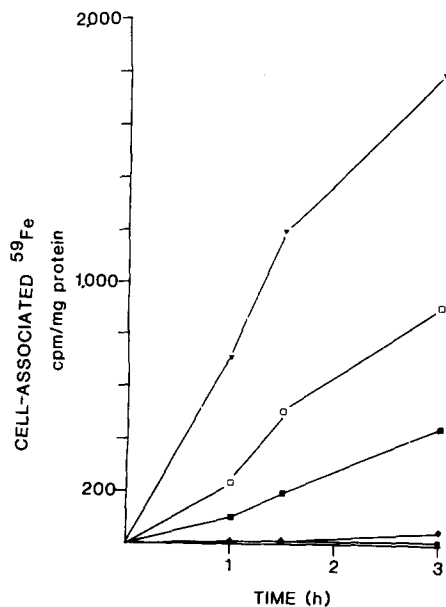
Figure 7. Accumulation of ^{59}Fe from Tf^{59}Fe$_2$ WTB ($\square$), TRVa ($\blacklozenge$), TRVb ($\bullet$), TRVa-11 ($\blacksquare$), and TRVb-1 ($\blacktriangledown$) were grown in six well plates. The monolayers were washed three times with med 2 and incubated at 37°C for the indicated times in med 2 containing 5 µg/ml Tf^{59}Fe$_2$ and 2 mg/ml ovalbumin. Cells were washed free of unbound ligand, and solubilized in 0.1 N NaOH and 0.1% Triton X-100. Cell-associated radioactivity was determined. The values shown have been corrected for nonspecific cell–associated ^{59}Fe by inclusion of a 500-fold excess of unlabeled differic Tf. The nonspecific cell-associated ^{59}Fe was constant over the times examined and was ~300 cpm/mg protein.

expressed on the different cell lines. This result documents that the human Tf-R can fulfill its biological role in the transfectants.

Discussion

By selection for resistance to two Tf-toxin conjugates, CHO cell mutants have been isolated that do not bind detectable amounts of Tf. The mutants exhibited at least a 100-fold increased resistance to the Tf-MspSA conjugate toxin. The mutant cells are specifically defective in Tf uptake and not pleotropically defective in endocytosis as shown by uptake of another ligand, α_2-macroglobulin. The increased resistance is due to a defect in Tf binding, as the mutants have retained sensitivity to DT, indicating that the cells are not blocked at the site of toxin action or defective in toxin entry. Because the mutant cells were passaged during the three rounds of selection it is not known whether these cell lines were independently derived or if they have arisen from a common parent. The mutation(s) in these cells are stable with no reversion in the phenotype observed after continual passage for 6 mo in the absence of selection.

The defect(s) responsible for the Tf-R–variant phenotype has not been determined. In our characterization of the mutants the Tf-R was necessarily detected by assaying for Tf binding because antibodies against the CHO receptor are not available. The observed phenotype could result from (*a*) a reduction in the number of functional cell surface Tf-R to <10% of wild type levels; (*b*) synthesis of receptors with a

reduced affinity for Tf; (*c*) no synthesis of the Tf-R. Further studies are required to distinguish among these possibilities.

A double-selection scheme was used anticipating that a significant number of cells would survive due to an alteration in the site of toxin action. Since RA chain (60S ribosomes target, reference 21) and DT A chain (elongation factor 2 target, reference 21) block protein synthesis at different sites, a double-selection scheme would eliminate those cells resistant due to loss of the target site. Although it is not known whether such an approach was necessary, the selection scheme yielded a high percentage of surviving cells of the desired Tf-R–variant phenotype (40% had no detectable binding). The remaining 60% of the surviving cells have not been characterized, and therefore it is not known why they survived the selection scheme.

The mutant cells have been used as recipients for the cDNA clone of the human Tf-R. Stable transfectants expressing the human receptor have been isolated. The Tf uptake pathway, as characterized by ^{125}I-Tf and f-Tf internalization in these cells, is very similar to uptake by the native Tf receptor in WTB cells. The transfected receptor also functions in the delivery of ^{59}Fe to cells as mediated by Tf^{59}Fe$_2$ uptake. These results demonstrate that the regions of the human receptor responsible for internalization, sorting, and recycling are recognized by CHO cells. In agreement with this result, the human insulin and epidermal growth factor receptors have been functionally expressed in CHO cells (5, 16).

Two laboratories have successfully expressed the human Tf-R in mouse L cells (17, 19). In both cases the genomic sequences were used for transfection, and the expression of the receptor was identified on the surface of the transfected cells using immunofluorescence. In one case the receptor was shown to be competent for Tf-mediated Fe uptake by using antibodies that specifically block Tf binding to the endogenous mouse Tf-R (19). However in neither study were the endocytic properties of the transfected receptor examined.

The expression of the human Tf-R in the Tf-R–variant cell lines should prove to be a powerful system for the further analysis of the structure–function relationship of the Tf-R, since in this system the functioning of the transfected receptor can be followed using the natural ligand.

References

1. Aisen, P., and I. Listowksy. 1980. Iron transport and storage proteins. *Annu. Rev. Biochem.* 49:367–393.
2. Casellas, P., B. J. P. Bourrie, P. Gros, and F. K. Jansen. 1984. Kinetics of cytotoxicity induced by immunotoxins. *J. Biol. Chem.* 259:9359–9364.
3. Colombatti, M., L. Greenfield, and R. J. Youle. 1986. Cloned fragment of diphtheria toxin linked to T cell-specific antibody identifies regions of B chain active in cell entry. *J. Biol. Chem.* 261:3030–3035.
4. Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 80:2258–2262.

5. Ebina, Y., M. Edery, L. Ellis, D. Standring, J. Beaudoing, R. A. Roth, and W. J. Rutler. 1985. Expression of functional human insulin receptor from a cloned cDNA in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA.* 82:8014–8018.

6. Enns, C. A., J. W. Larrick, H. Suomalainen, J. Schroder, and H. H. Sussman. 1983. Co-migration and internalization of transferrin and its receptor on K562 cells. *J. Cell Biol.* 97:579–585.

7. Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russel, and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* 1:11–39.

8. Hanover, C. J., and R. B. Dickson. 1985. Transferrin: receptor-mediated endocytosis and iron delivery. *In* Endocytosis. I. Pastan and M. C. Willingham, editors. Plenum Publishing Corp., New York. 131–162.

9. Harding, C., J. Hueser, and P. Stahl. 1983. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J. Cell Biol.* 97:329–399.

10. Hemmaplardh, D., and E. H. Morgan. 1974. Transferrin and iron uptake by human cells in culture. *Exp. Cell Res.* 887:107–212.

11. Hopkins, C. R., and Trowbridge, I. S. 1983. Internalization and processing of transferrin and transferrin receptor in human carcinoma cells A431. *J. Cell Biol.* 97:508–521.

12. Kingsley, D. M., and M. Krieger. 1984. Receptor-mediated endocytosis of low density lipoprotein: somatic cell mutants define multiple genes required for expression of surface-receptor activity. *Proc. Natl. Acad. Sci. USA.* 81:5454–5458.

13. Klausner, R. D., J. van Renswoude, G. Ashwell, C. Kempf, A. N. Schechter, A. Dean, and K. R. Bridges. 1983. Receptor-mediated endocytosis of transferrin in K562 cells. *J. Biol. Chem.* 258:4715–4724.

14. Klausner, R. D., J. van Renswoude, C. Kempf, K. Rao, J. L. Bateman, and A. R. Robbins. 1984. Failure to release iron from transferrin in a Chinese hamster ovary cell mutant pleiotropically defective in endocytosis. *J. Cell Biol.* 98:1098–1101.

15. Kuhn, L. C., A. McClelland, and F. H. Ruddle. 1984. Gene transfer, expression, and molecular cloning of the human transferrin receptor gene. *Cell.* 37:95–103.

16. Livneh, E., R. Prywes, O. Kashless, N. Reiss, I. Sasson, Y. Mory, A. Ullrich, and J. Schlessinger. 1986. Reconstitution of human epidermal growth factor receptor and its deletion mutants in cultured hamster cells. *J. Biol. Chem.* 261:12490–12497.

17. McClelland, A., L. C. Kuhn. and F. H. Ruddle. 1984. The human transferrin receptor gene: genomic organization, and the complete primary structure of the receptor deduced from a cDNA sequence. *Cell.* 39:267–274.

18. Miskimins, W. K., and N. Shimizu. 1981. Genetics of all surface receptors for bioactive peptides: variants of Swiss/3T3 fibroblasts resistant to cytotoxic chimeric insulin. *Proc. Natl. Acad. Sci. USA.* 78:445–449.

19. Newman, R., D. Domingo, J. Trotter, and I. Trowbridge. 1983. Selection and properties of a mouse L-cell transformant expressing human transferrin receptor. *Nature (Lond.).* 304:643–645.

20. Newman, R., C. Schneider, R. Sutherland, L. Vodinelich, and M. Greaves. 1982. The transferrin receptor. *Trend. Biochem. Sci.* 7: 396–400.

21. Olsnes, S., and K. Sandvig. 1983. Entry of toxic proteins into cells. *In* Receptor-mediated Endocytosis. P Cuatrecasas and J. Roth, editors. Chapman Hall, London/New York. 189–236.

22. Raso, V., and M. Basala. 1984. A highly cytotoxic human transferrin-ricin A chain conjugate used to select receptor-modified cells. *J. Biol. Chem.* 259:1143–1149.

23. Robbins, A. R., R. Myerowitz, R. J. Voule, G. J. Murry, and D. M. Neville, Jr. 1981. The mannose 6-phosphate receptor of CHO cells. *J. Biol. Chem.* 256:10618–10622.

24. Robbins, A. R., S. S. Peng, and J. L. Marshall. 1983. Mutant Chinese hamster ovary cells pleiotropically defective in receptor-mediated endocytosis. *J. Cell Biol.* 96:1064–1071.

25. Schneider, C., M. J. Owen, D. Banville, and J. G. Williams. 1984. Primary structure of the human transferrin receptor deduced from the mRNA sequence. *Nature (Lond.).* 311:675–678.

26. Southern, P. J., and P. J. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV_{40} early region promoter. *J. Mol. Appl. Genet.* 1:327–341.

27. Stuchbury, T., M. Shipton, R. Norris, J. P. Malthous, and S. Brucklehurst. 1975. A reporter group delivery system with both absolute and selective specificity for thiol groups and an improved fluorescent probe containing the 7-nitrobenzo-z-oxa 1,3-diazole moiety. *Biochem. J.* 151:417–432.

28. Thompson, L. H., and R. M. Baker. 1973. Isolation of mutants in mammalian cells. *Methods Cell Biol.* 6:209–281.

29. Willingham, M. C., S. A. Hanover, R. B. Dickerson, and I. Pastan. 1984. Morphological characterization of the pathway of transferrin endocytosis and recycling in human KB cells. *Proc. Natl. Acad. Sci. USA.* 81:175–179.

30. Yamashiro, D. J., B. Tycko, S. R. Fluss, and F. R. Maxfield. 1984. Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell.* 37:789–800.