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Intermolecular disulfide bonds are not required for the expression of the dimeric state and functional activity of the transferrin receptor

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The human transferrin receptor is expressed as a disulfide-linked dimer at the cell surface. The sites of intermolecular disulfide bonds are Cys-89 and Cys-98. We have examined the functional significance of the covalent dimeric structure of the transferrin receptor by substitution of Cys-89 and Cys-98 with serine residues. Wild-type and mutated transferrin receptors were expressed in Chinese hamster ovary cells (clone TF^-) that lack detectable endogenous transferrin receptors. The rates of receptor endocytosis and recycling were measured and the accumulation of iron by cells incubated with [^{59}Fe]diferric transferrin was investigated. No significant differences between these rates were observed when cells expressing wild-type and mutated receptors were compared. The structure of the mutant receptor lacking intermolecular disulfide bonds was investigated. The presence of a population of mutant receptors with a non-covalent dimeric structure was indicated by cross-linking studies using diferric [^{125}I]transferrin and the bifunctional reagent disuccinimidyl suberimidate. However, sucrose density gradient sedimentation analysis of Triton X-100 solubilized transferrin receptors demonstrated that the mutant receptor existed as a monomer in the absence of diferric transferrin and as an apparent dimer in the presence of this receptor ligand. We conclude that the covalent dimeric structure of the transferrin receptor is not required for the expression of the dimeric state and functional activity of the receptor.

Key words: disulfide bonds/human transferrin receptor

Introduction

The uptake of iron by cells is mediated by the endocytosis of diferric transferrin bound to specific cell surface receptors (for review see May and Cautrecasas, 1985). The transferrin receptor has been characterized as a disulfide-linked homodimer composed of 90 kd subunits (Sutherland *et al.*, 1981; Omary and Trowbridge, 1981a,b). In previous studies we (Davis and Meisner, 1987) and others (Jing and Trowbridge, 1987; McGraw *et al.*, 1987, 1988; Rothenberg *et al.*, 1987; Zerial *et al.*, 1987) have investigated the effect of specific alterations in the structure of the receptor on function. The approach used was to perform site-directed mutagenesis of the human transferrin receptor cDNA and to express the wild-type and mutated cDNAs in murine cells (Davis and Meisner, 1987; Rothenberg *et al.* 1987; Zerial *et al.*, 1987). A problem with these studies is that because the receptor is a disulfide-linked dimer, the transfected cells express a mixture of human and murine homodimeric and heterodimeric receptors. The presence of the three distinct receptor species has been identified using species-specific anti-transferrin receptor monoclonal antibodies (Newman *et al.*, 1983; Davis and Meisner, 1987).

One approach that has been taken to overcome the problems of interpretation that result from the presence of a mixed receptor population composed of disulfide-linked dimers at the cell surface has been described (Davis and Meisner, 1987). The human transferrin receptor was expressed at a high level in murine 3T3 fibroblasts. The cells were then incubated with an IgM antimurine transferrin receptor monoclonal antibody. This procedure was effective at down-regulating endogenous murine transferrin receptors (both murine homodimers and murine–human heterodimers) and the cells obtained expressed only human homodimeric receptors. However, an objection to the use of this down-regulation procedure is that it is not known what effects this treatment may have on the properties of the cells. The difficulty of interpretation of experiments that involve the expression of the human transferrin receptor cDNA in normal murine cells has therefore led to the development of an alternative strategy. The use of cells that are deficient in transferrin receptor expression for the transfection studies would resolve the problems caused by the dimeric structure of the receptor outlined above. Recently, McGraw *et al.* (1987) have described the isolation of Chinese hamster ovary (CHO) cells that lack functional transferrin receptors and their use for the expression of the human transferrin receptor cDNA in the absence of endogenous receptors.

The functional significance of the dimeric state of the receptor is not understood. The purpose of the study reported here was to investigate whether the intermolecular disulfide bonds that cause the covalent association of the transferrin receptor as a dimer are required for the function of the receptor. Recently, Jing and Trowbridge (1987) have reported that the sites of intermolecular disulfide bonding are Cys-89 and Cys-98. In the present study this information was used to evaluate the role of the intermolecular disulfide bonds. We performed site-directed mutagenesis of the transferrin receptor cDNA to replace these cysteine residues with serine. Wild-type (Cys-89, Cys-98) and mutated (Ser-89, Ser-98) human transferrin receptors were expressed in cultured CHO cells that lack functional transferrin receptors. Stable cell lines were obtained and the properties of the wild-type covalent dimeric receptors were compared with mutant receptors that do not form covalent dimers. It was observed that the intermolecular disulfide bonds between monomeric subunits were not required for the expression of transferrin receptor function.

Results

Isolation of TF⁻ cells

In a previous study we investigated the properties of the human transferrin receptor expressed in Swiss 3T3 fibroblasts (Davis and Meisner, 1987). Therefore, in initial attempts to isolate cells that lack functional transferrin receptors we mutagenized Swiss 3T3 fibroblasts with ethylmethanesulfonate and selected for cells deficient in transferrin receptors using a diferric transferrin–ricin A chain conjugate. This procedure was not successful for obtaining a mutant cell line without transferrin receptors. The reason for the lack of success may be due to the inefficiency of the selection method used. Alternatively, it is possible that the loss of expression of transferrin receptors may be a lethal mutation. This is because it has previously been shown that the expression of the transferrin receptor is co-ordinately regulated with cell growth and that monoclonal antibodies that bind to the receptor inhibit cell growth (Trowbridge and Lopez, 1982; Trowbridge *et al.*, 1982; Lesley and Schulte, 1984, 1985). These considerations indicate that the transferrin receptor may be required for the growth of murine 3T3 fibroblasts and that it may not be possible to isolate a mutant cell line without transferrin receptors.

It has been demonstrated that mutant CHO cells which fail to release iron from diferric transferrin are viable (Klausner *et al.*, 1984). This information suggests that the loss of transferrin receptor expression by CHO cells may not be a lethal mutation. In further experiments we therefore used CHO cells. After mutagenesis and selection with a transferrin-toxin conjugate, a clone of CHO cells (designated TF^-) was obtained that lacks functional transferrin receptors. Control experiments demonstrated that TF^- cells did not accumulate radioactivity when incubated with [^{59}Fe]diferric transferrin (Figure 1). Furthermore, no specific binding of diferric [^{125}I]transferrin was observed to TF^- cells or to isolated membranes prepared from these cells (data not shown). Recently, similar mutant CHO cells have been reported by McGraw *et al.* (1987).

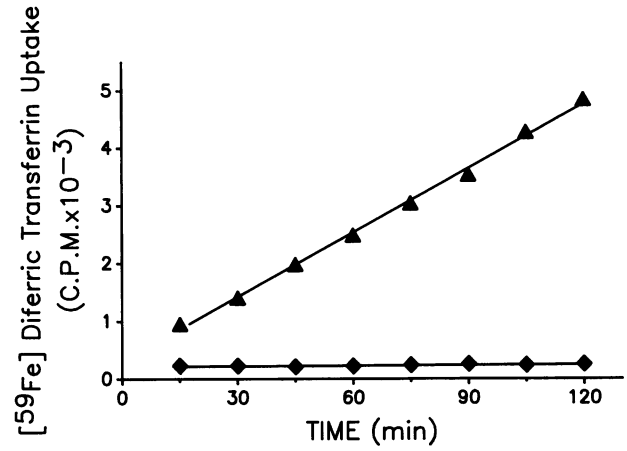

Fig. 1. Accumulation of [^{59}Fe]diferric transferrin by CHO cells. CHO cells were seeded in 16 mm wells and incubated at 37°C with 300 nM [^{59}Fe]diferric transferrin. After defined times the cells were washed rapidly at 4°C and the radioactivity associated with the cells was determined using a Beckman liquid scintillation counter. The accumulation of radioactivity by parental CHO-K1 cells (▲) and by TF^- cells (◆) was investigated. The results presented are the means of triplicate observations. Similar results were observed in three separate experiments.

Expression of the human transferrin receptor in TF⁻ cells

Wild-type and mutated forms of the human transferrin receptor were expressed in TF^- cells. Figure 2 shows the results of the analysis of the structure of transferrin receptors immunoprecipitated from [^{35}S]methionine-labeled cells. No transferrin receptors were observed in control TF^- cells. However, cells transfected with the wild-type transferrin receptor cDNA were observed to express a protein that was immunoprecipitated by an anti-transferrin receptor antibody which migrated during polyacrylamide gel electrophoresis with an apparent molecular mass of 180 kd. In the presence of reductant the major molecular species observed was 90 kd. These data are consistent with the disulfide-linked homodimeric structure of the wild-type receptor. In contrast to these observations, it was observed that the Ser-89 and Ser-98 receptor migrated as a monomer (90 kd) in the presence and absence of reductant (Figure 2). We conclude that the substitution of Cys-89 and Cys-98 with serine residues causes the loss of the intermolecular disulfide bonds (Jing and Trowbridge, 1987). In a control experiment it was observed that the substitution of serine for a cysteine residue that is not involved in disulfide bond formation, Cys-62, did not alter the covalent dimeric structure of the receptor (Figure 2).

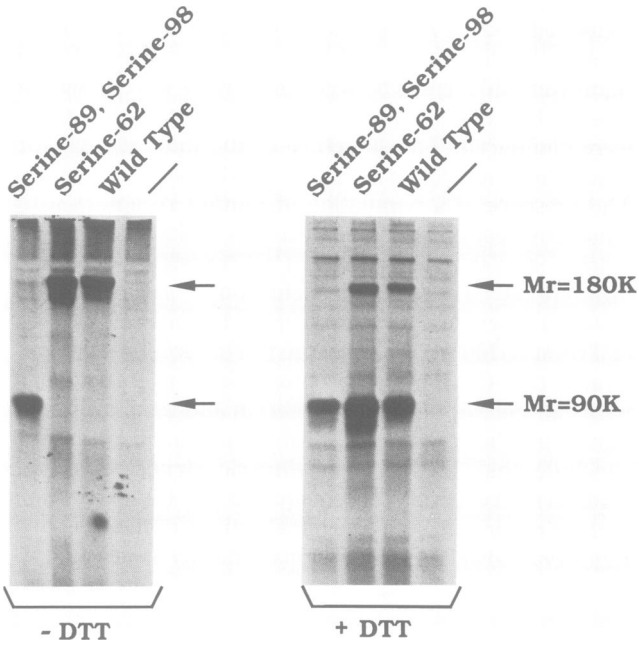

Fig. 2. Investigation of the covalent dimeric state of the transferrin receptor. CHO cells were labeled by incubation for 18 h with 10 μM [^{35}S]methionine (50 μCi/ml). The transferrin receptors were isolated by immunoprecipitation and analyzed by polyacrylamide gel electrophoresis in the presence and absence of DTT (50 mM). The figure presents an autoradiograph of the dried gel.

The ligand binding properties of the wild-type and Ser-89,Ser-98 transferrin receptors expressed in TF^- cells were investigated by the analysis of the diferric [^{125}I]transferrin binding isotherm. The data obtained were analyzed by the method of Scatchard (Figure 3). Linear plots were obtained indicating the presence of a single class of high affinity binding sites expressed at the surface of the

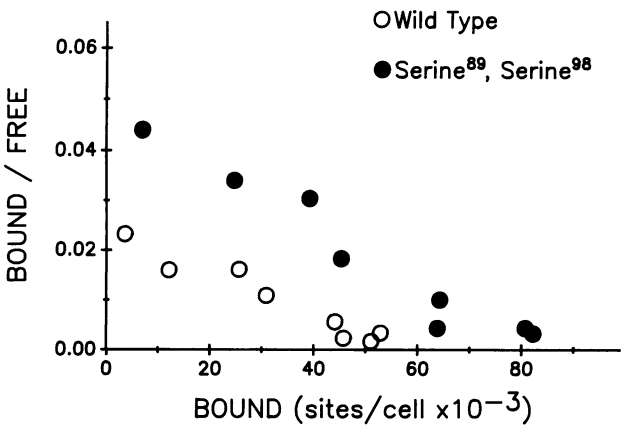

Fig. 3. Analysis of the diferric [^{125}I]transferrin binding isotherm. TF$^-$ cells expressing wild-type (○) and Ser-89,Ser-98 transferrin receptors (●) were seeded in 16 mm wells and grown to confluence. The specific binding of diferric [^{125}I]transferrin to cell surface receptors was measured at 4°C. The data obtained was analyzed using the computer program EBDA (Elsevier Biosoft) and is presented by the method of Scatchard (1949). The affinity of the receptors was estimated to be 1.6 nM (53 000 per cell) and 1.3 nM (84 000 per cell) for WT and Ser-89,Ser-98 transferrin receptors, respectively.

cells. The K_d was estimated to be 1.6 nM (53 000 sites per cell) and 1.3 nM (84 000 sites per cell) for wild-type and Ser-89,Ser-98 transferrin receptors, respectively.

Comparison of the kinetics of cycling of the wild-type and Ser-89,Ser-98 transferrin receptors

To compare the kinetics of endocytosis of the wild-type and mutated transferrin receptors the apparent first order rate constants for endocytosis and recycling were measured. Figure 4 shows the results obtained for the analysis of the internalization of diferric [^{125}I]transferrin by the In/Sur method described by Wiley and Cunningham (1982). This method involves the measurement of the rate of intracellular accumulation of ligand under conditions where the number of occupied cell surface receptors is constant and no release of accumulated ligand occurs. A linear rate of diferric [^{125}I]transferrin uptake with time was observed up to ~8 min. After this time a sharp decrease in the rate of accumulation of [^{125}I]transferrin occurred because of the release of accumulated transferrin as apotransferrin (Figure 4). The apparent first order endocytotic rate constant was calculated to be 0.29 ± 0.019 min^{-1} and 0.28 ± 0.032 min^{-1} (mean ± standard deviation, n = 3) for wild-type and Ser-89,Ser-98 receptors, respectively. The rate of transferrin receptor recycling was estimated by measuring the rate of release of [^{125}I]apotransferrin from cells (Figure 5). The apparent first order rate constant for recycling was calculated to be 0.101 ± 0.025 min^{-1} and 0.069 ± 0.015 min^{-1} (mean ± standard deviation, n = 3) for cells expressing wild-type and Ser-89,Ser-98 receptors, respectively. The first order rate constants for endocytosis and recycling of wild-type and Ser-89,Ser-98 receptors are summarized in Table I.

The experiments performed to investigate the cycling of the transferrin receptor employed diferric [^{125}I]transferrin as a probe for the function of the receptor (Figures 3–5). To examine the endocytosis of the receptor in the absence of diferric transferrin, [^{125}I]Fab fragments of a monoclonal antibody (OKT9) were prepared. This antibody binds to an epitope on the extracellular domain of the receptor and is suitable as a receptor ligand to investigate the internalization of the receptor in the absence of diferric transferrin.

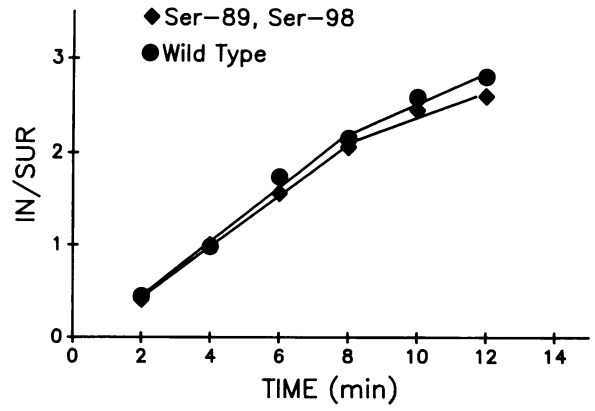

Fig. 4. Endocytosis of diferric [^{125}I]transferrin. The endocytosis of diferric [^{125}I]transferrin was investigated using the In/Sur method of Wiley and Cunningham (1982) as described under Materials and methods. The results shown are the means of determinations made in three separate experiments. The first-order endocytotic rate constant was estimated to be 0.29 ± 0.019 min^{-1} and 0.28 ± 0.032 min^{-1} for wild-type (●) and Ser-89,Ser-98 (◆) transferrin receptors, respectively (mean ± SD, n = 3).

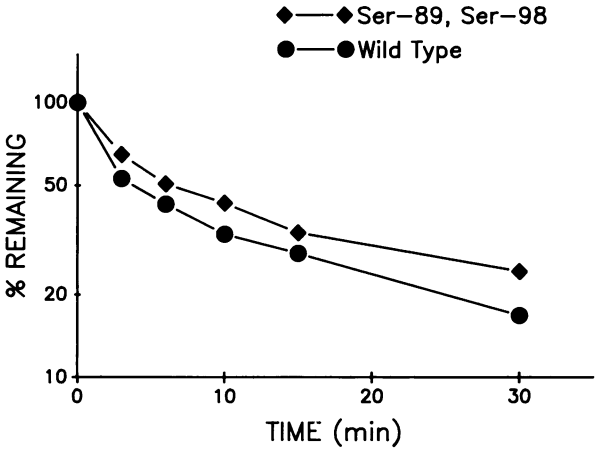

Fig. 5. Recycling of [^{125}I]apotransferrin. The release of [^{125}I]apotransferrin by CHO cells was measured as described under Materials and methods. The results represent the means of determinations made in three separate experiments. The apparent first-order rate constants for [^{125}I]apotransferrin release were calculated using the computer program ENZFITTER (Elsevier Biosoft) and are estimated to be 0.101 ± 0.025 min^{-1} and 0.69 ± 0.015 min^{-1} for TF$^-$ cells expressing wild-type (●) and Ser-89,Ser-98 (◆) receptors, respectively (mean ± SD, n = 3).

Table I. Summary of kinetic rate constants

	Apparent first order rate constant (min^{-1})	
	Wild-type receptor Cys-89,Cys-98	Mutated receptor Ser-89,Ser-98
Endocytosis	0.29 ± 0.019	0.28 ± 0.032
Recycling	0.101 ± 0.025	0.069 ± 0.015

The first-order rate constants for the endocytosis and recycling of the transferrin receptor were estimted from the rates of the internalization of diferric [^{125}I]transferrin (Figure 4) and release of [^{125}I]apotransferrin (Figure 5), respectively. The results represent the mean ± SD of measurements made in three separate experiments.

Incubation of cells expressing Ser-89,Ser-98 and wild-type receptors at 37°C resulted in the rapid accumulation [^{125}I]Fab fragments. Washing the cells at pH 3.0 (4°C) to cause dissociation of the cell surface bound [^{125}I]Fab fragments indicated that the cells accumulated the [^{125}I]Fab fragments into an intracellular compartment. We conclude that the wild-type and Ser-89,Ser-98 receptors are competent for endocytosis in the absence of diferric transferrin (not shown).

To examine the function of the transferrin receptors expressed in TF$^-$ cells the accumulation of radioactivity by cells incubated with [^{59}Fe]diferric transferrin was measured. Figure 1 shows that untransfected TF$^-$ cells do not accumulate iron when incubated with diferric transferrin, consistent with the absence of detectable receptors present in these cells. In contrast, TF$^-$ cells transfected with the wild-type human transferrin receptor cDNA rapidly accumulated transferrin-bound iron (Figure 6). This data indicates that the expressed human receptor is able to restore transferrin receptor function in TF$^-$ cells. The accumulation of radioactivity by TF$^-$ cells incubated with [^{59}Fe]diferric transferrin was also observed after transfection with the Ser-89,Ser-98 transferrin receptor. A larger amount of iron uptake was observed by cells expressing Ser-89,Ser-98 receptors compared with cells with wild-type receptors. The difference in uptake could be caused by either a difference in the properties of these receptors or by the greater expression of cell surface receptors (Figure 3) in cells expressing Ser-89,Ser-98 receptors (84 000 per cell) compared with cells with wild-type receptors (53 000 per cell). Therefore, in order to compare the rate of iron uptake, the data obtained were normalized to the accumulation of [^{59}Fe]diferric transferrin per cell surface receptor (Figure 6). The rate of iron accumulation was rapid and no significant difference between the results obtained for wild-type and Ser-89,Ser-98 receptors was observed.

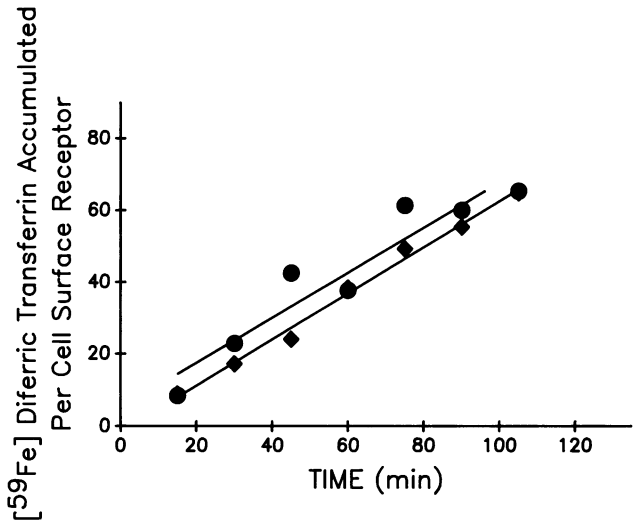

Fig. 6. Accumulation of [^{59}Fe]diferric transferrin. TF$^-$ cells and cells expressing wild-type (●) and Ser-89,Ser-98 (◆) transferrin receptors were seeded in 16 mm wells and incubated for different times with 300 nM [^{59}Fe]diferric transferrin. The cells were then washed and the radioactivity associated with the cells was measured using a Beckman liquid scintillation counter. In parallel experiments the number of cell surface binding sites for diferric [^{125}I]transferrin was determined at 4°C by Scatchard analysis of the binding isotherm (Figure 3). The results are presented as the moles of [^{59}Fe]diferric transferrin accumulated per mole of cell surface receptors. The data presented are the means of triplicate determinations.

Examination of the oligomeric structure of the Ser-89,Ser-98 transferrin receptor

Although the Ser-89,Ser-98 transferrin receptor lacks intermolecular disulfide bonds and migrates as a monomeric protein on SDS–polyacrylamide gels (Figure 2) a significant question remains concerning whether the protein exists in the absence of SDS as a monomer or as a non-covalently associated dimer. Experiments were therefore designed to investigate the oligomeric structure of the Ser-89,Ser-98 transferrin receptor. In initial experiments an experimental strategy using the bifunctional cross-linking agent disuccinimidyl suberimidate (DSS) was employed to examine whether the Ser-89,Ser-98 receptor exists as a non-covalently associated dimer. CHO cells were labeled with [^{35}S]-methionine, incubated with 0.2 mM DSS at 4°C for 15 min, and the transferrin receptors were isolated by immunoprecipitation. The cross-linked complexes were analyzed by polyacrylamide gel electrophoresis and fluorography. The Ser-89,Ser-98 receptor was observed as a 90 kd monomeric protein, but after cross-linking with DSS a small amount of a 180 kd dimeric receptor species was observed (not shown). Similar results were obtained with the wild-type receptor (not shown). These data indicate that the Ser-89,Ser-98 transferrin receptor can form a non-covalently associated dimeric structure. However, as the cross-linking of the receptor as a dimeric species was inefficient, the data obtained do not allow conclusions to be drawn about the relative distribution of Ser-89,Ser-98 receptors between monomeric and dimeric states.

The oligomeric structure of the Ser-89,Ser-98 transferrin receptor bound to diferric [^{125}I]transferrin was investigated by covalent cross-linking analysis using DSS. CHO cells expressing wild-type and Ser-89,Ser-98 transferrin receptors were metabolically depleted in order to inhibit receptor endocytosis and were incubated at 4°C with diferric [^{125}I]transferrin. The cells were washed at 4°C and cross-linking was initiated by the addition of 0.2 mM DSS for 15 min. The major cross-linked complexes observed were 260 kd and 170 kd for wild-type and Ser-89,Ser-98 receptors, respectively (Figure 7). After reduction with DTT a major complex of 170 kd was observed for both wild-type and mutant receptors. Together these data indicate that the 170 kd complex corresponds to [^{125}I]transferrin cross-linked to a monomeric receptor and that the 260 kd complex corresponds to a dimeric receptor. Figure 7 shows that there was a low efficiency of cross-linking of [^{125}I]transferrin with dimeric Ser-89,Ser-98 receptors (260 kd complex). A higher efficiency of cross-linking of Ser-89,Ser-98 receptors to obtain a 260 kd complex was observed if the [^{125}I]transferrin was bound to the cells at 37°C prior to cross-linking at 4°C (Figure 7). No effect of temperature was observed on the cross-linking of the wild-type transferrin receptor (Figure 7).

The examination of the Ser-89,Ser-98 transferrin receptor by cross-linking analysis suggests the hypothesis that transferrin regulates the oligomeric structure of the mutant receptor. To test this hypothesis, further experiments were designed to investigate the structure of the Ser-89,Ser-98 receptor using a method based on different physical principles. Experiments were performed to investigate the

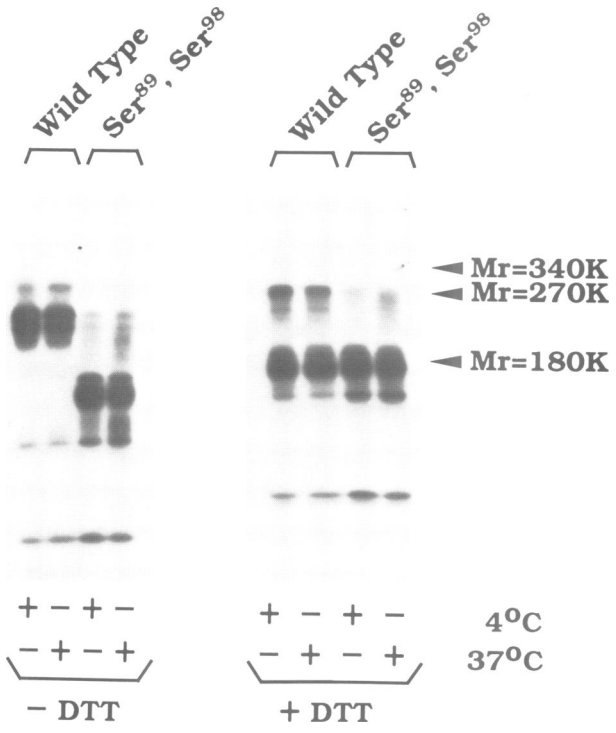

Fig. 7. Covalent cross-linking analysis of diferric [^{125}I]transferrin binding to wild-type and Ser-89,Ser-98 transferrin receptors. TF$^-$ cells expressing wild-type and Ser-89,Ser-98 transferrin receptors were grown in 35 mm dishes. The cells were metabolically depleted by incubation with 20 mM 2-deoxyglucose and 10 mM NaN_3 in order to inhibit receptor-mediated endocytosis. The cells were then incubated at 37°C or at 4°C with 5 nM diferric [^{125}I]transferrin for 3 h. The cells were washed with cold medium and cross-linking was initiated by the addition of 0.2 mM DSS for 15 min at 4°C. The cross-linking reaction was terminated and the transferrin receptors were isolated by immunoprecipitation. The cross-linked complexes were analyzed by polyacrylamide gel electrophoresis in the presence and absence of DTT (50 mM) to reduce disulfide bonds. Cross-linked phosphorylase (Sigma) was used as molecular weight standards for electrophoresis. The figure presents an autoradiograph of the dried gel. Similar results were obtained in three separate experiments.

sucrose density gradient sedimentation properties of the Triton X-100 solubilized receptors. It was observed that the wild-type transferrin receptor sedimented faster than the Ser-89,Ser-98 receptor during sucrose density gradient centrifugation. The sedimentation coefficients for the wild-type and Ser-89,Ser-98 receptors were calculated to be 7.5 S and 5.1 S, respectively (Table II). In contrast, it was observed that the wild-type and Ser-89,Ser-98 receptors bound to diferric transferrin could not be resolved using this technique (Figure 8B). The sedimentation coefficients of the wild-type and mutant receptors in the presence of diferric transferrin was calculated to be 9.0 S (Table II). As the wild-type receptor is a disulfide-linked dimer these data suggest that the Ser-89,Ser-98 receptor is a monomeric protein under the conditions of the experiment and that the binding of diferric transferrin causes the Ser-89,Ser-98 receptor to sediment as an apparent dimer.

Experiments were performed to investigate whether apotransferrin regulates the state of aggregation of the Ser-89,Ser-98 transferrin receptor. As apotransferrin binds to the receptor at low pH, the sucrose density gradient sedimentation properties of wild-type and Ser-89,Ser-98

Table II. Summary of sucrose density gradient sedimentation coefficients

	Wild-type transferrin receptor	Ser-89,Ser-98 transferrin receptor
pH 7.4		
control	7.5 S	5.1 S
+ diferric transferrin	9.0 S	9.0 S
pH 5.0		
control	9.0 S	9.0 S
+ apotransferrin	9.6 S	9.6 S

The sucrose density gradient sedimentation properties of wild-type and mutant transferrin receptors were investigated in the presence and absence of transferrin at pH 7.4 and pH 5.0 as described in the legends to Figures 8 and 9. The sedimentation coefficients of the receptors were calculated by comparison with the sedimentation properties of protein standards: catalase (11 S), rabbit IgG (7.1 S), bovine serum albumin (4.3 S) and ovalbumin (3.7 S) as described under Materials and methods.

receptors were investigated at pH 5.0. Figure 9A shows that wild-type and Ser-89,Ser-98 transferrin receptors co-sedimented on sucrose gradients. Similar results were obtained in the presence of apotransferrin (Figure 9B). The sedimentation coefficients of the receptors in the absence and presence of apotransferrin at pH 5.0 were calculated to be 9.0 S and 9.6 S, respectively (Table II). As the wild-type receptor in the presence of apotransferrin (Figure 9B) is a disulfide-linked dimer bound to two ligand molecules with a predicted molecular mass of 340 kd, the rapid sedimentation of the wild-type receptor (180 kd) in the absence of apotransferrin (Figure 9A) suggests that the wild-type receptor exists in an aggregated state at low pH. In the presence of transferrin, the apparent aggregation of receptors at low pH was not observed (Figure 9).

Discussion

Expression of the human transferrin receptor in TF$^-$ cells

The CHO clone TF$^-$ described in this report does not express detectable high affinity transferrin receptors. The mutation induced by ethylmethanesulfonate that caused this phenotype has not been characterized in detail. As transferrin receptor function can be restored to the cells by transfection with the human cDNA, we conclude that this mutation is recessive. Possible causes of the phenotype of the TF$^-$ cells include (i) a marked reduction or complete loss of the biosynthesis of the receptor and (ii) the synthesis of receptors with an extremely low affinity for diferric transferrin. The results obtained in the present study do not allow the rigorous distinction between these alternative explanations and further studies are required to characterize the phenotype of the TF$^-$ cells. The absence of binding of diferric transferrin to TF$^-$ cells allows the examination of the properties of human receptors expressed in these cells. TF$^-$ cells are therefore useful for the comparison of the properties of wild-type and mutated transferrin receptors. In the present study, the accumulation of iron by TF$^-$ cells incubated with diferric transferrin was reconstituted by the expression of human transferrin receptors in these cells (Figures 1 and 6).

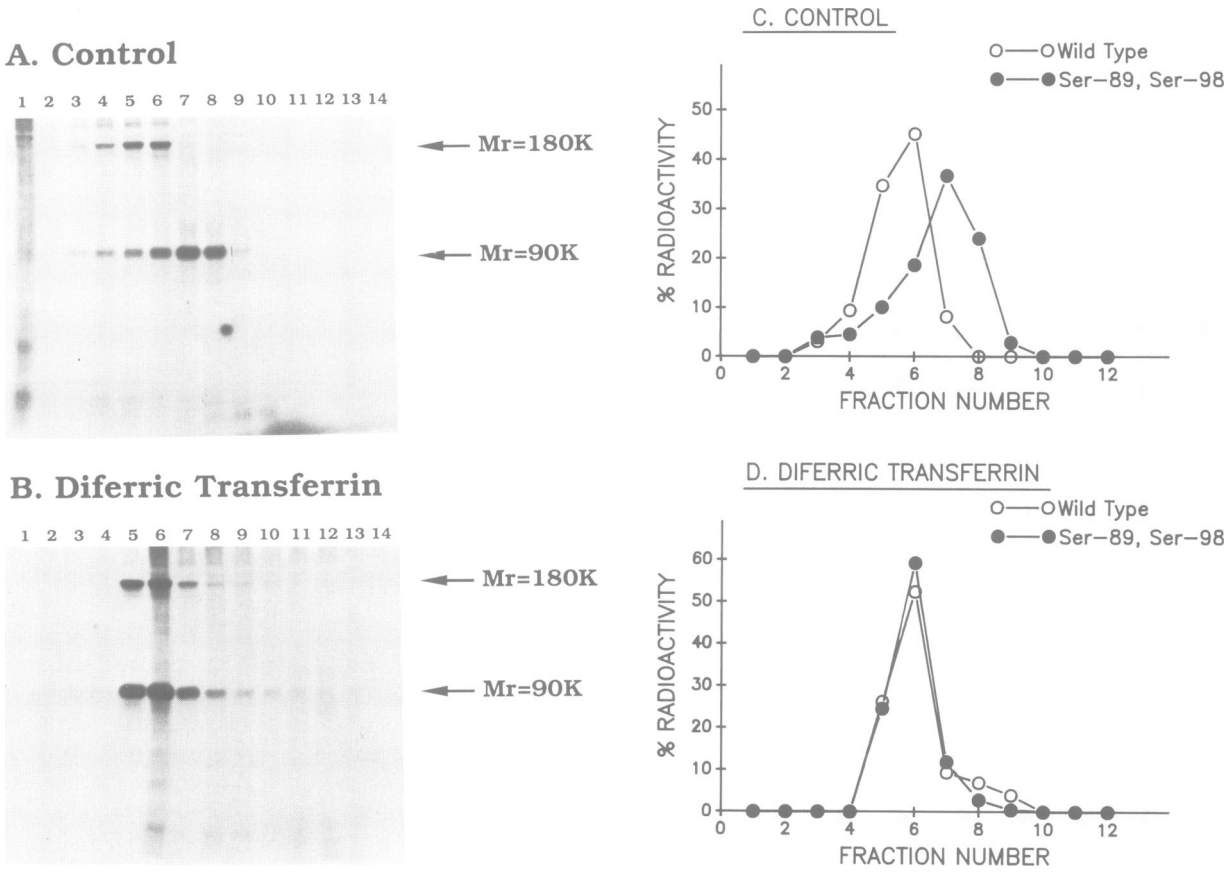

Fig. 8. Sucrose density gradient sedimentation analysis of mutant and wild-type transferrin receptors in the presence and absence of diferric transferrin. CHO cells labeled with [^{35}S]methionine were incubated in the absence (**A**,**C**) and presence (**B**,**D**) of 100 nM diferric transferrin for 60 min at 37°C. The cells were then solubilized in 0.1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 30 mM Hepes (pH 7.4). Insoluble material was removed by centrifugation for 30 min at 100 000 *g* (4°C). A sample of solubilized wild-type receptor (100 μl) and Ser-89,Ser-98 receptor (100 μl) were mixed together and layered onto a 5–25% linear sucrose gradient and centrifuged for 24 h (A and C) and for 18 h (B and D). Experiments using cells incubated with diferric transferrin were performed using solubilization buffer and sucrose gradients that contained 100 nM diferric transferrin. After centrifugation 12 fractions were taken from each tube and numbered consecutively from the bottom of the centrifuge tube. The receptors were isolated from each fraction by immunoprecipitation and analyzed by polyacrylamide gel electrophoresis (non-reduced) and fluorography. Wild-type and Ser-89,Ser-98 receptors were identified with molecular masses of 180 kd and 92 kd, respectively. Panels A and B present fluorograms of the polyacrylamide gels obtained. The radioactivity associated with the wild-type and Ser-89,Ser-98 receptors in each fraction was measured by excising the bands from the gel and liquid scintillation counting (C and D). Similar results were obtained in three separate experiments.

Comparison of the wild-type transferrin receptor with a mutant receptor lacking intermolecular disulfide bonds

Analysis of the diferric [^{125}I]transferrin binding isotherm indicated the presence of a single class of binding sites expressed at the surface of TF$^-$ cells with K_d = 1.6 nM and 1.3 nM for wild-type and Ser-89,Ser-98 receptors, respectively (Figure 3). These data indicate that the loss of intermolecular disulfide bonds resulting from the substitution of the cysteine residues with serine does not cause a marked alteration in the affinity of the receptor. The lack of effect of the mutation on the binding of transferrin is consistent with previous observations that the receptor ligand binding site can be isolated in functional form as a tryptic fragment of the receptor (Omary and Trowbridge, 1981a; Bleil and Bretscher, 1982; Schneider *et al.*, 1982) and is therefore stable to structural alterations to distal regions of the receptor.

Comparison of the kinetics of endocytosis of diferric [^{125}I]transferrin demonstrated that there was no significant difference between the rates measured for TF$^-$ cells expressing wild-type and Ser-89,Ser-98 receptors (Figure 4). Recycling of transferrin receptors was estimated from the release of [^{125}I]apotransferrin by cells. The mean rate of release was slower for the Ser-89,Ser-98 receptor (0.069 ± 0.015 min^{-1}; mean ± SD) than for the wild-type receptor (0.101 ± 0.025 min^{-1}) but the difference was not statistically significant (Figure 5). In further experiments the accumulation of iron by cells incubated with [^{59}Fe]diferric transferrin was investigated. It was observed that there was no significant difference between the rate of accumulation of iron by cells expressing wild-type and Ser-89,Ser-98 receptors (Figure 6) consistent with the similar kinetic properties of the cycling of these receptors (Figures 4 and 5). Together these data indicate that the mutations causing the loss of the intermolecular disulfide bonds caused no significant change in the functional properties of the transferrin receptor.

The transferrin receptor intermolecular disulfide bonds are dispensible for the function of the receptor (Figures 3–6). A consequence of this conclusion is that the role of the covalent dimeric structure of the receptor can be questioned.

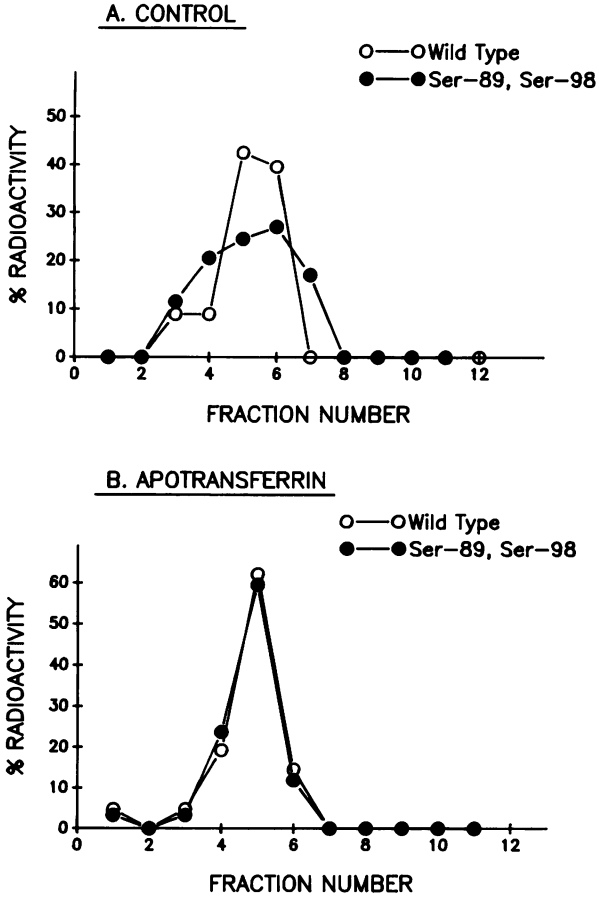

Fig. 9. Effect of low pH on the sucrose density gradient sedimentation of wild-type and Ser-89,Ser-98 receptors. The sucrose density gradient sedimentation analysis of wild-type and Ser-89,Ser-98 receptors was investigated at pH 5.0. CHO cells labeled with [^{35}S]methionine were incubated in the absence (**A**) and presence (**B**) of 100 mM apotransferrin for 60 min at 37°C in 120 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 30 mM MES (pH 5.0). The cells were then solubilized in 0.1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 30 mM Mes (pH 5.0) and insoluble material was removed by centrifugation for 30 min at 100 000 *g* (4°C). Samples containing wild-type (100 μl) and Ser-89,Ser-98 receptors (100 μl) were mixed and layered onto a 5–25% linear sucrose gradient (pH 5.0). Experiments using cells incubated with apotransferrin were performed using solubilization buffer and sucrose gradients that contained 100 nM apotransferrin. After centrifugation for 18 h (4°C) the gradients were fractionated and the 12 fractions obtained were numbered consecutively from the bottom of the tube. The receptors were immunoprecipitated from each fraction and analyzed by polyacrylamide gel electrophoresis (non-reduced) and fluorography. The wild-type (180 kd) and Ser-89,Ser-98 (90 kd) receptors were excised from the gel and the associated radioactivity was measured by liquid scintillation counting. The figure presents the results obtained in the absence (A) and presence (B) of 100 nM apotransferrin. Similar results were obtained in three separate experiments.

However, the data presented here do not allow the conclusion to be drawn that the dimeric structure of the receptor is not required for functional activity. This is because the Ser-89,Ser-98 receptor was able to associate non-covalently as a dimer. Evidence for a dimeric structure was provided by cross-linking studies. The Ser-89,Ser-98 receptor could be cross-linked using DSS as a dimer with [^{125}I]transferrin and was observed as a 260 kd complex on polyacrylamide gels (Figure 7). The efficiency of cross-linking of the dimeric Ser-89,Ser-98 receptor was very low, but was increased by the incubation of the cells at 37°C with diferric [^{125}I]transferrin prior to cross-linking at 4°C. The lower efficiency of cross-linking observed after the binding of diferric [^{125}I]transferrin at 4°C suggests that in the presence of diferric [^{125}I]transferrin there is a temperature-sensitive change in the conformation of the receptor. As the cells were metabolically depleted, the effect of temperature was not caused by receptor internalization at 37°C (Ciechanover *et al.*, 1983; Davis *et al.*, 1987). A hypothesis that can account for the cross-linking data obtained is that the binding of diferric transferrin to the Ser-89,Ser-98 receptor stabilizes the non-covalent dimeric structure of the receptor and that this process is inhibited during incubation at 4°C compared with 37°C. At 4°C the rate of lateral diffusion of the receptor molecules in the membrane would be expected to be slow and may consequently limit the level of cross-linked receptor dimers observed.

In order to test the hypothesis that transferrin stabilizes the non-covalent dimeric state of the Ser-89,Ser-98 receptor, experiments were designed to investigate the oligomeric structure of the receptor using a method based on different physical principles. It was observed that the Triton X-100 solubilized Ser-89,Ser-98 receptor sedimented slower than the wild-type receptor during sucrose density gradient centrifugation analysis in the absence of diferric transferrin (Figure 8). As the wild-type receptor is a disulfide-linked dimer, the data obtained indicate that the Ser-89,Ser-98 receptor may be a monomer under the conditions of this experiment. However, in the presence of diferric transferrin the wild-type and mutant receptors could not be resolved by sucrose density gradient centrifugation analysis (Figure 8). To account for this data it is necessary to propose that diferric transferrin causes a conformational change in the Ser-89,Ser-98 receptor. This proposed conformational alteration may be associated with the aggregation of the receptor. The co-sedimentation of the wild-type and the mutant receptor in the presence of diferric transferrin indicates that the Ser-89,Ser-98 receptor may be a dimer under these conditions. A non-covalent dimeric state of the Ser-89,Ser-98 receptor is consistent with a recent report that the gel filtration properties of a 70 kd tryptic fragment of the receptor indicate a dimeric structure (Turkewitz *et al.*, 1988a).

Apotransferrin binds with high affinity to the transferrin receptor at the low pH observed in acidified endosomes. Experiments were therefore performed to investigate the effects of apotransferrin on the oligomeric state of the transferrin receptor at pH 5.0. It was observed that the sucrose density gradient sedimentation properties of wild-type and Ser-89,Ser-98 receptors were similar in the presence and absence of apotransferrin (Figure 9, Table II). These data were unexpected because the predicted molecular masses of the wild-type and Ser-89,Ser-98 receptors in the absence (180 kd and 90 kd, respectively) and presence (340 kd and 170 kd, respectively) of apotransferrin indicate that these species should have been resolved by sucrose density gradient centrifugation. The similar sedimentation in the presence and absence of apotransferrin suggests that the receptors aggregate at low pH and that the binding of apotransferrin inhibits this process. The data obtained are consistent with an apparent dimeric structure for the wild-type receptor and an apparent tetrameric structure for the Ser-89,Ser-98 receptor at pH 5.0 in the absence of

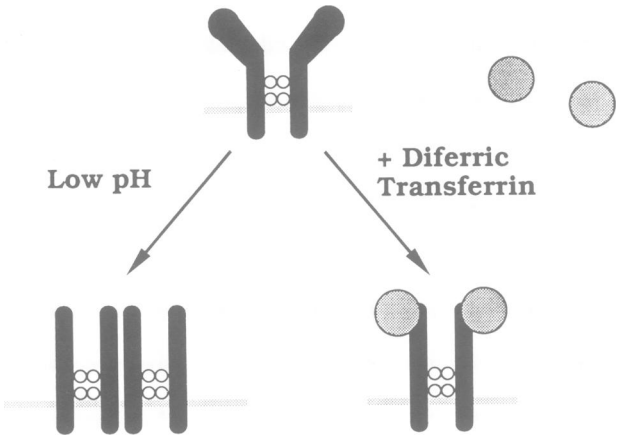

Fig. 10. Schematic representation of the regulation of transferrin receptor structure by low pH and by diferric transferrin.

apotransferrin (Figure 9, Table II). In the presence of apotransferrin, the data obtained are consistent with an apparent dimeric structure for both the wild-type and the Ser-89,Ser-98 receptor (Figure 9, Table II). The apparent oligomeric properties of the wild-type and mutant receptors observed (Figures 8 and 9, Table II) are consistent with those recently reported for a 70 kd tryptic fragment of the transferrin receptor which forms extensive aggregates after prolonged incubation at low pH (Turkewitz *et al.*, 1988b).

Together, the data presented here indicate that the Ser-89,Ser-98 transferrin receptor is expressed as a mixed population of oligomeric states. Diferric transferrin bound to the receptor stabilizes an aggregated state of the receptor. It was observed that the Triton X-100 solubilized Ser-89, Ser-98 receptor sediments as an apparent dimer in the presence of diferric transferrin during sucrose density gradient centrifugation (Figure 8). The stabilization of the apparent dimeric structure of the Ser-89,Ser-98 receptor suggests that diferric transferrin causes a change in the conformation of the receptor which increases the interaction between the subunits of the receptor. A further conformational alteration of the receptor was caused by low pH in the absence of transferrin (Figure 9). Figure 10 presents a schematic representation of the proposed effects of transferrin and the exposure to low pH on the conformation of the wild-type transferrin receptor.

We conclude that the transferrin receptor intermolecular disulfide bonds are dispensible for the expression of the dimeric state and the functional activity of the receptor. However, the dimeric structure of the transferrin receptor may be significant for the function of the receptor. The hypothesis that a dimeric receptor structure is required for the expression of the functional properties of the receptor requires rigorous examination. As other cell surface receptors have been shown to exist in a dimeric form [for example, the receptors for low density lipoprotein (van Dreil *et al.*, 1987) and epidermal growth factor (Yarden and Schlessinger, 1987)] the general question of the significance of dimeric receptor states is important for the further understanding of the mechanism of receptor function.

Materials and methods

Materials

Na[^{125}I], [^{35}S]methionine, [^{35}S]dATP and [^{59}Fe]Cl_3 were from Amersham. Human transferrin was from Behring Diagnostics and was saturated with iron as described (Davis and Czech, 1986). Diferric [^{125}I]transferrin (5–7 Ci/g) and [^{59}Fe]diferric transferrin (30–40 mCi/g) were prepared as described (Davis and Czech, 1986). The monoclonal antibody OKT9 was purified by protein A–Sepharose chromatography from the tissue culture supernatant of hybridoma cells obtained from the American Type Culture Collection. Fab fragments of OKT9 were prepared by papain digestion using reagents purchased from Pierce Chemical Company and used according to the manufacturer's directions. The papain digestion mixture was applied to a protein A–Sepharose column and the flow-through fractions were collected. The Fab fragments in the flow-through fractions were further purified by gel filtration chromatography. The Fab fragments were iodinated by the iodogen method (Pierce) to a specific activity of 5–12 Ci/g according to the manufacturer's directions. The [^{125}I]Fab fragments were isolated by Sephacryl S-200 chromatography. Enzymes were from Boehringer and Amersham except for Sequenase which was obtained from United States Biochemical Corp. Diferric transferrin–ricin A chain conjugate was prepared from ricin A chain (Vector Laboratories) and diferric transferrin using *N*-succinimidyl 3-(2-pyridyldithio) proponate (Pharmacia) as described (Raso and Basala, 1984).

Plasmid construction

The human transferrin receptor cDNA (McClelland *et al.*, 1984; Kuhn *et al.*, 1984) was obtained from Dr F.Ruddle (Yale University). A *Bam*HI–*Hin*dIII 0.9 kb fragment of the cDNA (which contains the 5′ untranslated region and the coding region for the cytoplasmic domain) was sub-cloned into M13mp18 as described (Davis and Meisner, 1987). Site-directed mutagenesis of Cys-62 (TGT), Cys-89 (TGT) and Cys-98 (TGT) was carried out according to Zoller and Smith (16) using 20-mer oligonucleotides coding for serine (5′-CCA AAA AGG TCG AGT GGA AG-3′;5′-G GGC TAT TCG AAA GGG GTA G-3′ and 5′-CA AAA ACT GAG TCG GAG AGA-3′). Mutations were confirmed by sequencing using [^{35}S]dATP, ddNTPs and Sequenase (Sanger *et al.*, 1977). RF DNA was isolated and the cDNA fragment prepared by endonuclease digestion and agarose gel electrophoresis. This fragment was ligated to a *Hin*dIII–*Bgl*II 1.9 kb fragment of the receptor cDNA that contains the coding region for the carboxyl terminal domain of the receptor and the full length cDNA was isolated by agarose gel electrophoresis as described (Davis and Meisner, 1987). The full-length wild-type and mutant cDNAs were then cloned into the expression vector pX (obtained from Dr G.Johnson, National Jewish Center, Denver) using standard techniques (Maniatis *et al.*, 1982). This vector contains the mouse dihydrofolate reductase gene as a selectable marker and allows the expression of the transferrin receptor cDNA by utilizing the SV40 early promoter and polyadenylation signals. The correct orientation of the cDNAs cloned into the expression vector was determined by restriction endonuclease digestion.

Tissue culture

CHO-K1 cells were obtained from the American Type Culture Collection and were maintained in Ham's F12 medium supplemented with 5% fetal bovine serum (Gibco). A clone of CHO-K1 cells without transferrin receptors was obtained after mutagenesis using ethylmethanesulfonate as described by McGraw *et al.* (1987). A confluent 75 cm^2 flask of CHO cells was incubated in medium for 24 h with 300 μg/ml of ethylmethanesulfonate. The cells were then washed and passaged twice over 7 days. The cells were then incubated with 20 nM diferric transferrin–ricin A chain conjugate in serum-free Ham's F12 medium supplemented with 50 nM monensin and 1 nM insulin-like growth factor 1 (Amgen) for 24 h. The cells were then fed with medium containing 5% fetal bovine serum and allowed to grow to confluence. The cells were split 1:2 and reselected with the transferrin–ricin A chain conjugate. The efficiency of the selection process was investigated in control experiments using wild-type CHO-K1 cells and TRVA cells obtained from Dr F.R.Maxfield (Columbia University). It was observed that the toxin conjugate caused 50% of the wild-type cells to die after incubation for 24 h. Therefore, in order to obtain a cell population enriched with cells lacking functional transferrin receptors, it was necessary to repeat the selection process several times. After six rounds of selection, the cells were seeded at low density and 89 colonies were isolated using cloning rings. The clones were expanded, seeded in 16 mm wells and screened for the specific binding of 10 nM diferric [^{125}I]transferrin at 4°C. From this screening one clone (designated TF$^-$) was isolated that exhibited no specific binding of diferric [^{125}I]transferrin. In order to confirm that the TF$^-$ cells do not express functional transferrin receptors three further experiments were performed. First, it was observed that the cells were resistant to the diferric transferrin–ricin A chain conjugate (data not shown). Second, the cells did not accumulate radioactivity when incubated with [^{59}Fe]diferric transferrin (Figure 1). Third, membranes were isolated from the cells (Davis and Czech, 1985) and the specific binding of diferric

[^{125}I]transferrin was assessed as described (Davis *et al.*, 1986). No specific binding of diferric [^{125}I]transferrin to the membranes was found (data not shown). We conclude from these data that the TF$^-$ cells do not express a functional transferrin receptor.

Human transferrin receptors were expressed in TF$^-$ cells by transfection of the cells using the calcium phosphate method. Stable colonies resistant to 500 nM amethopterin (Sigma) in MEMα medium supplemented with 5% dialyzed fetal bovine serum were obtained and isolated using cloning rings. Cells expressing wild-type transferrin receptors were designated WT. Cells expressing the mutant transferrin receptor in which both Cys-89 and Cys-98 were replaced with serine residues were designated Ser-89,Ser-98. Cells expressing transferrin receptors in which Cys-62 was replaced with a serine residue were designated Ser-62.

Binding of diferric [^{125}I]transferrin to cell surface receptors

Binding assays were performed on cells grown in 16 mm wells. The cells were washed with serum-free medium and incubated for 30 min at 37°C in 120 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM Hepes (pH 7.4), and 30 μM bovine serum albumin. The medium was removed and rapidly replaced with medium at 4°C. Diferric [^{125}I]transferrin was added to the cells which were incubated for 180 min at 4°C. The monolayers were washed three times with cold medium and solubilized with 900 μl of 1 M NaOH. Radioactivity associated with the cells was quantitated with a Beckman gamma counter. Non-specific binding was estimated in incubations with a 200-fold excess of unlabeled ligand.

Transferrin receptor endocytosis

The endocytotic rate constant for transferrin receptor internalization was measured by the In/Sur method described by Wiley and Cunningham (1982). The method involves the measurement of the rate of intracellular accumulation of ligand under conditions where the number of occupied cell surface receptors is constant and no release of accumulated ligand occurs. CHO cells were incubated at 37°C with 10 nM diferric [^{125}I]transferrin. At defined times the binding of the [^{125}I]transferrin to the cells was measured by rapidly washing the cell monolayers at 4°C and determining the associated radioactivity with a gamma counter. Cell surface and intracellularly bound [^{125}I]transferrin was determined by incubation of the cells for 3 min at 4°C with 50 mM NaCl, 150 mM glycine (pH 3.0). Intracellular [^{125}I]transferrin was estimated by measurment of the cell-associated radioactivity after acid washing (Haigler *et al.*, 1980; Lamb *et al.*, 1983). Cell surface bound [^{125}I]transferrin was estimated by subtraction of the intracellularly bound [^{125}I]transferrin from the total specific binding of [^{125}I]transferrin observed to cell monolayers. Non-specific binding of [^{125}I]transferrin was estimated in incubations containing a 200-fold excess of unlabeled ligand.

Endocytosis by the transferrin receptor in the absence of diferric transferrin was investigated using [^{125}I]Fab fragments of the monoclonal antibody OKT9 as a ligand for the transferrin receptor. CHO cells were seeded in 16 mm wells and grown to a density of 5×10^4 cells per well. The cells were washed with serum-free medium and incubated for 30 min at 37°C in 1 ml of 120 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM Hepes (pH 7.4). The cells were then transferred to 0.5 ml of medium containing 10 nM [^{125}I]Fab fragments. At defined times the binding of [^{125}I]Fab fragments to the cells was measured by rapidly washing the cell monolayers at 4°C with medium and determining the radioactivity associated with the cells using a gamma counter. Cell surface and intracellularly bound [^{125}I]Fab fragments were determined by incubation of the cells for 3 min at 4°C with 1 ml of 150 mM glycine (pH 3.0), 50 mM NaCl. Intracellular [^{125}I]Fab fragments were estimated by measurement of the cell-associated radioactivity after the acid washing procedure (Haigler *et al.*, 1980; Lamb *et al.*, 1983). Control experiments demonstrated that the acid washing treatment caused the dissociation of 95% of the specific cell surface bound [^{125}I]Fab fragments at 4°C. Non-specific binding of [^{125}I]Fab fragments was estimated in incubations containing a 200-fold excess of unlabeled ligand.

Transferrin receptor recycling

The rate constant for the recycling of transferrin receptors was estimated by measuring the rate of recycling of [^{125}I]apotransferrin using a pulse−chase technique. CHO cells were seeded in 16 mm wells and grown to a density of 5×10^4 cells per well. The cells were then incubated with 10 nM diferric [^{125}I]transferrin for 2 h at 37°C. The medium was then removed and the cells were washed at 0°C and subsequently incubated for 3 min at 0°C with 50 mM NaCl, 150 mM glycine (pH 3.0) to remove ligand bound to the cell surface (Haigler *et al.*, 1980; Lamb *et al.*, 1983). The medium was then removed and the cells were incubated at 37°C to initiate recycling for defined times. The radioactivity remaining associated with the cells was measured with a gamma counter.

Accumulation of [^{59}Fe]diferric transferrin

CHO cells were seeded in 16 mm wells and grown to a density of 5×10^4 cells per well. The cells were washed with serum-free medium and incubated for 30 min at 37°C in 120 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM Hepes (pH 7.4) and 30 μM bovine serum albumin. The cells were then incubated for different times with 300 nM [^{59}Fe]diferric transferrin at 37°C. At defined times the cells were washed rapidly at 4°C, solubilized and the radioactivity associated with the cells was measured with a liquid scintillation counter as described (Davis and Czech, 1986).

Analysis of the expression of the transferrin receptor

CHO cells were seeded in 22 mm wells and grown to a density of 1×10^5 cells per well. The cells were then transferred to medium containing 10 μM [^{35}S]methionine (50 μCi/ml) for 18 h. The transferrin receptors were then immunoprecipitated from the solubilized cells and analyzed by polyacrylamide gel electrophoresis and fluorography as described (Davis and Meisner, 1987).

Sucrose density gradient centrifugation

Samples analyzed by sucrose density gradient centrifugation were dissolved in 0.1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 30 mM Hepes (pH 7.4). Insoluble material was removed by centrifugation at 100 000 *g* for 30 min at 4°C. The samples (200 μl) were then layered on a 5−25% (w/v) linear sucrose gradient prepared using the solubilization buffer (4.2 ml final volume). In some experiments the gradients also included 100 nM diferric transferrin or apotransferrin. Experiments designed to investigate the effect of low pH were performed by the substitution of Hepes (pH 7.4) in the solutions with 2(*N*-morpholino)ethanesulfonic acid (Mes) (pH 5.0). The sucrose gradients were centrifuged in a Beckman 50.1 rotor at 40 000 r.p.m. at 4°C for 18−24 h. After centrifugation the gradients were fractionated and the 12 fractions obtained were numbered consecutively from the bottom of the tube to the top. The sedimentation of the transferrin receptor was compared with that of protein molecular weight standards: catalase (11 S), rabbit IgG (7.1 S), bovine serum albumin (4.3 S) and ovalbumin (3.7 S). Aliquots of the fractions obtained from the gradients were analyzed by polyacrylamide gel electrophoresis and the protein standards were identified by staining the gel with Coomassie blue. After 18 h of centrifugation the locations of the protein standards in the sucrose gradients were: catalase (fractions 4 and 5), IgG (fractions 6 and 7), albumin (fraction 8) and ovalbumin (fractions 9 and 10). After 24 h of centrifugation the locations were: catalase (fraction 3), IgG (fractions 5 and 6), albumin (fractions 7 and 8) and ovalbumin (fraction 9). The observed sedimentation properties of the standard proteins were then used to calculate the Svedberg coefficients for the transferrin receptor complexes.

Analysis of covalent cross-linking of receptors

CHO cells were seeded in 22 mm wells and grown to a density of 1×10^5 cells per well. The cells were then transferred to medium containing 10 μM [^{35}S]methionine (100 μCi/ml) for 18 h. The cell monolayers were washed three times with serum-free medium and incubated at 37°C for 30 min. The cells were then cooled to 4°C and transferred to 1 ml of 120 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM Hepes (pH 7.4) and 0.2 mM DSS. After 15 min of incubation at 4°C the cells were washed and then solubilized in 1 ml of 1% Nonidet P-40, 0.5 M NaCl, 5 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 25 mM Tris (pH 8.0). The solubilized cells were centrifuged at 100 000 *g* for 30 min at 4°C and the supernatant fraction was used for the isolation of transferrin receptors by immunoprecipitation with the monoclonal antibody OKT9 as described (Davis and Meisner, 1987). Cross-linked receptors were analyzed by linear gradient (3−9%) polyacrylamide gel electrophoresis and fluorography.

Metabolic depletion of TF$^-$ cells

TF$^-$ cells were washed with serum-free medium (120 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 30 μM bovine serum albumin 25 mM Hepes, pH 7.4) and incubated for 30 min at 37°C in medium supplemented with 20 mM 2-deoxyglucose and 10 mM NaN_3 as described (Ciechanover *et al.*, 1983; Davis *et al.*, 1987). It has been reported that this metabolic depletion causes the loss of detectable transferrin receptor internalization by HepG2 hepatoma cells (Ciechanover *et al.*, 1983) and by A431 epidermoid carcinoma cells (Davis *et al.*, 1987). Control experiments were performed to confirm that the metabolic depletion caused the inhibition of transferrin receptor internalizaion by TF$^-$ cells. Metabolically depleted TF$^-$ cells expressing wild-type or Ser-89,Ser-98 transferrin receptors were incubated at 37°C with 5 nM diferric [^{125}I]transferrin. Intracellular

accumulation of [^{125}I]transferrin was estimated by measurement of the cell-associated radioactivity after acid washing (Haigler *et al.*, 1980; Lamb *et al.*, 1983). No significant accumulation of radioactivity by the cells was observed after acid washing. We conclude that metabolic depletion of TF^- cells causes an inhibition of the internalization of transferrin receptors.

Analysis of covalent cross-linking of [^{125}I]transferrin

Assays were performed using TF^- cells grown in 35 mm wells. The cells were metabolically depleted by incubation for 30 min with 20 mM 2-deoxy-glucose and 10 mM NaN_3 as described above. The cells were then incubated at 37°C or at 4°C for 3 h with 5 nM diferric [^{125}I]transferrin (1 ml). The monolayers were then washed three times with cold medium and subsequently incubated in albumin-free medium (1 ml) containing 0.2 mM DSS for 15 min at 4°C. The monolayers were washed twice (4°C) and solubilized in 1 ml of 1% Nonidet P-40, 0.5 M NaCl, 1 mM PMSF, 10 μg/ml leupeptin, 5 mM EGTA, 25 mM Tris (pH 8.0). The solubilized cells were centrifuged at 100 000 *g* for 30 min (4°C) and the supernatant fraction was used for the isolation of transferrin receptors by immunoprecipitation with the monoclonal antibody OKT9 as described (Davis and Meisner, 1987). Cross-linked transferrin receptor complexes were analyzed by polyacrylamide (3–9% linear gradient) gel electrophoresis and autoradiography.

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