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Identification of the intermolecular disulfide bonds of the human transferrin receptor and its lipid-attachment site

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Structural studies of the human transferrin receptor have shown that the molecule is a disulfide-bonded dimer consisting of two identical subunits (M_r = 95 000) which are post-translationally modified by the addition of a fatty acyl moiety. Oligonucleotide site-directed mutagenesis has been used to obtain mutant molecules in which each of the four cysteines, residues 62, 67, 89 and 98, clustered within or adjacent to the membrane-spanning region were modified to serine. By first preparing mutants with only one of these cysteine residues modified to serine and then obtaining additional mutants in which different combinations of two cysteine residues were modified, we have shown that both cysteine 89 and cysteine 98, which are located in the extracellular domain of the receptor, are involved in intermolecular disulfide bonds. Further, we have identified cysteine 62 as the major site of acylation. Each of the mutant molecules is synthesized and transported to the cell surface when the modified human transferrin receptor cDNAs are transiently expressed in simian Cos cells. It should therefore now be possible to design experiments to determine whether these modified receptors bind transferrin normally and mediate iron uptake.

Key words: site-directed mutagenesis/transferrin receptor

Introduction

The transferrin receptor binds the serum transport protein, transferrin and facilitates iron uptake into the cell via receptor-mediated endocytosis (reviewed in Newman *et al.*, 1982; Trowbridge *et al.*, 1984; Dautry-Varsat and Lodish, 1984). The expression of the transferrin receptor is coordinately regulated with cell growth, and monoclonal antibodies that bind to the receptor and block its function inhibit cellular proliferation (Trowbridge and Lopez, 1982; Trowbridge *et al.*, 1982; Lesley and Schulte, 1984,1985). The transferrin receptor has been structurally characterized and is a disulfide-bonded dimer consisting of two identical subunits (M_r = 95 000) (Omary and Trowbridge, 1981a,b). The receptor is glycosylated and further post-translationally modified by the addition of one or more fatty acyl groups and by phosphorylation (Omary and Trowbridge, 1981a,b; Schneider *et al.*, 1982). The primary structure of the human transferrin receptor has been deduced from the nucleotide sequence of its cDNA (Schneider *et al.*, 1984; McClelland *et al.*, 1984). The receptor is 760 amino acids in length and consists of a carboxy-terminal extracellular domain of 670 residues, a hydrophobic transmembrane-spanning region, and an amino-terminal cytoplasmic domain of 61 amino acids. The functional significance of neither the dimeric nature of the receptor nor its acylation is known. The human transferrin receptor contains eight cysteine residues. Tryptic cleavage of the receptor on the cell surface indicates that only four of these residues, cysteines 62, 67, 89 and 98, which are clustered within or adjacent to the hydrophobic transmembrane spanning region, are candidates for involvement in intermolecular disulfide bonds (Omary and Trowbridge, 1981a,b; Schneider *et al.*, 1982; Schneider *et al.*, 1984; McClelland *et al.*, 1984). On the basis of the pH dependence of the nucleophilic cleavage of the lipid moiety from the transferrin receptor (Omary and Trowbridge, 1981a), and by analogy with vesicular stomatitis virus (VSV) transmembrane glycoprotein (Rose *et al.*, 1984) and HLA-B7 and HLA-DR antigens (Kaufman *et al.*, 1984), it is likely that the fatty acid moiety is attached via a thioester bond. Tryptic cleavage experiments indicate that if cysteine were the site of attachment then the lipid moiety must be linked to one or more of the four cysteine residues clustered around the transmembrane-spanning region. To determine which cysteines are involved in intermolecular disulfide bond formation and which are the lipid attachment sites, we have used oligonucleotide site-directed mutagenesis to modify the nucleotide sequence of the human transferrin receptor cDNA so as to convert each of the four cysteine residues to serine. Transient expression of the modified cDNAs in African green monkey Cos cells, followed by immunoprecipitation and SDS–polyacrylamide gel electrophoresis of the human transferrin receptor metabolically labeled with either [^{35}S]methionine or [^{3}H]palmitate, has permitted the identification of the cysteine residues involved in intermolecular disulfide bonds and also the major lipid attachment site.

Results

Construction of mutants

The strategy for the construction of the human transferrin receptor mutants is outlined in Figure 1. The 1.1-kb *Bam*HI–*Hin*dIII fragment isolated from pcDTR-1 (McClelland *et al.*, 1984) encoding the 5′ untranslated region and the first 237 residues of the human transferrin receptor (see Figure 1b) was cloned into M13mp19. Oligonucleotide site-directed mutagenesis was performed using the oligonucleotides shown in Figure 1a. Mutants were isolated as described in Materials and methods. The *Bam*HI–*Hin*dIII fragment containing the mutation was re-isolated from M13mp19 and together with the *Hin*dIII–*Bgl*II fragment of the human transferrin receptor isolated from pcDTR-1 was cloned into the *Bam*HI site of the expression vector JC119. The human transferrin receptor cDNA insert thus contained the entire coding region for the human transferrin receptor (Figure 1c). Preliminary experiments in which the wild-type human transferrin receptor *Bam*HI–*Bgl*II cDNA fragment was cloned into the JC119 vector in the correct orientation and then transfected into Cos cells showed that the human transferrin receptor was expressed on the surface of ~0.2–2% of the transfected cells. Thus, the entire 3′ untranslated region of the human transferrin receptor cDNA was not required for expression. Control experiments showed that B3/25 monoclonal antibody could be used to specifically detect the human transferrin receptor without

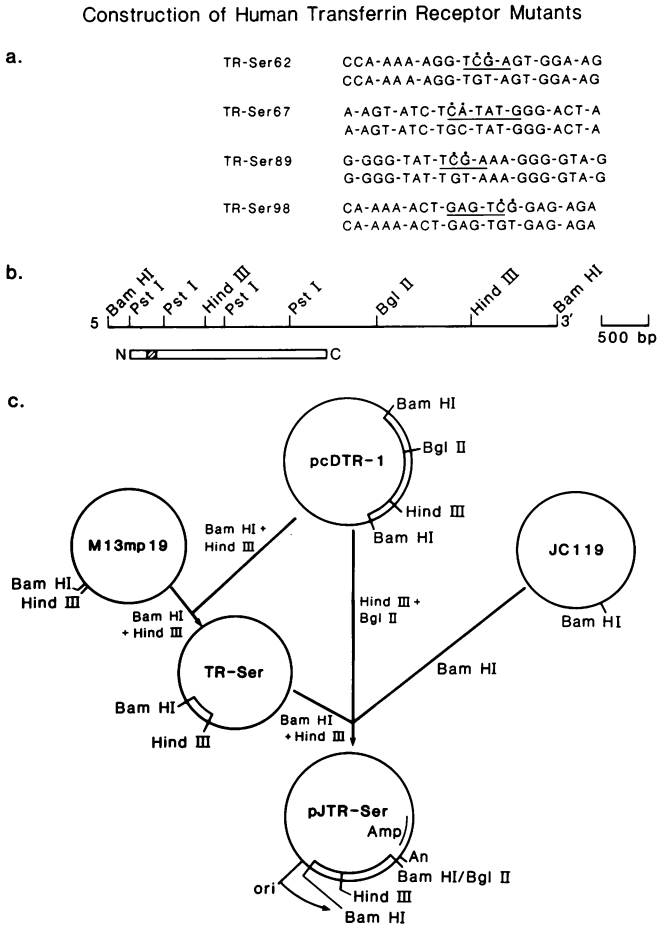

Fig. 1. Construction of human transferrin receptor mutants. **(a)** Comparison of the sequences of synthetic oligonucleotides used for site-directed mutagenesis of the human transferrin receptor cDNA (upper) and the sequences of the region of the human transferrin receptor cDNA (lower) from which each was derived. Each 20-mer contains two base mismatches (indicated by dots) with the nucleotide sequences surrounding the codons for cysteines 62, 67, 89, 98 of the receptor, respectively. In addition to converting each cysteine to serine, the nucleotide substitutions introduce a new restriction site (underlined sequences) in the cDNA (*Taq*I for TR-Ser 62 and TR-Ser 89, *Nde*I for TR-Ser 67, and *Hin*fI for TR-Ser 98). **(b)** Partial restriction map of the human transferrin receptor cDNA insert from pcDTR-1 plasmid (from data given in McClelland *et al.*, 1984). Also shown is the transferrin receptor protein aligned with the coding region of the cDNA. The hatched area represents the putative transmembrane spanning region. **(c)** Schematic representation of the protocol used to prepare human transferrin receptor mutants. As described in the text, the *Bam*HI−*Hin*dIII fragment from the 5′-end of the human transferrin receptor cDNA was cloned into M13mp19 and oligonucleotide site-directed mutagenesis performed as described in Materials and methods. The *Bam*HI−*Hin*dIII fragment containing the mutation was then cut out and together with the *Hin*dIII−*Bgl*II fragment from pcDTR-1 (see b) cloned into the *Bam*HI site of the expression vector, JC119. The orientation of the insert was determined by digestion with *Pst*I.

cross-reaction with the endogenous transferrin receptor of the Cos cells. Further, metabolic labeling of the transfected cells with either [^{35}S]methionine or [^{3}H]palmitate showed that sufficient amounts of human transferrin receptor were synthesized to allow detection by immunoprecipitation and SDS−polyacrylamide gel electrophoresis.

Identification of the cysteine residues of the human transferrin receptor involved in intermolecular disulfide bonds

In the initial experiments, four independent mutants were constructed in which each of the four cysteine residues at positions

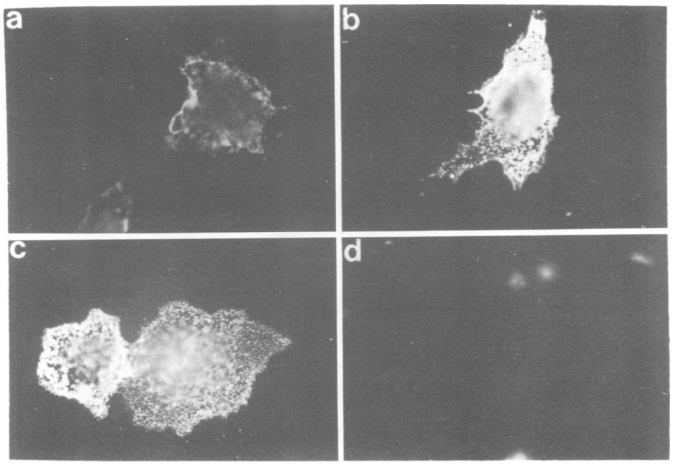

Fig. 2. Expression of human transferrin receptor mutants on the surface of Cos cells transfected with transferrin receptor cDNAs in the JC119 expression vector. Cos cells were transfected with JC119 constructs and 40−48 h later subjected to indirect immunofluorescence staining to detect the surface expression of the human transferrin receptor using B3/25 monoclonal antibody as described in Materials and methods. Shown are Cos cells transfected with **(a)** pJTR-Ser 98 (encoding serine at residue 98 instead of cysteine), **(b)** pJTR-Ser 98, 89 (encoding serines at residues 89 and 98), **(c)** pJTR (encoding the wild-type receptor) and **(d)** JC119 (negative control). Approximately 0.2−2% of the Cos cells transfected with pJTR and all the mutant constructs gave positive staining for the human transferrin receptor comparable to the examples shown. No staining was observed in controls in which the first stage antibody, B3/25, was omitted.

62, 67, 89 and 98 were separately modified to serine. In all cases, the mutant cDNAs were expressed in Cos cells, and the modified human transferrin receptor could be detected by indirect immunofluorescence on the cell surface of viable cells (Figure 2). The results of metabolic labeling experiments with [^{35}S]methionine are shown in Figure 3. The human transferrin receptor was immunoprecipitated from lysates of the transfected Cos cells and analyzed by SDS−polyacrylamide gel electrophoresis under reducing and non-reducing conditions. As a positive control, Cos cells transfected with a JC119 vector containing the wild-type *Bam*HI−*Bgl*II human transferrin receptor cDNA insert in the correct orientation were used. As a negative control, immunoprecipitates were prepared from Cos cells transfected with JC119 plasmid DNA alone. As shown in Figure 3, left-hand panel, when analyzed under non-reducing conditions, two of the mutants in which cysteine 62 and cysteine 67 were converted to serine respectively, gave identical results to the wild-type construction. Thus, each of these mutant molecules migrated as a species with an M_r = 190 000. However, the other two mutants gave different results. Although most of the human transferrin receptor modified by substitution of either cysteine 89 or cysteine 98 to serine migrated as a disulfide-bonded dimer (M_r = 190 000), a minor fraction of the receptors run under non-reducing conditions migrated with an apparent M_r of 95 000 (Figure 3, left-hand panel). This suggested that there were two intermolecular disulfide bonds between the two subunits of the receptor involving cysteine 89 and cysteine 98. Thus, we interpreted the small amounts of monomeric receptor seen under non-reducing conditions when one of these cysteines was modified to serine to indicate that loss of one intermolecular disulfide bond reduced the probability of formation of the remaining disulfide linkage. To test this hypothesis, double mutants were made in which cysteine 98 was converted to serine together with one of the other three cysteine residues clustered around the transmem-

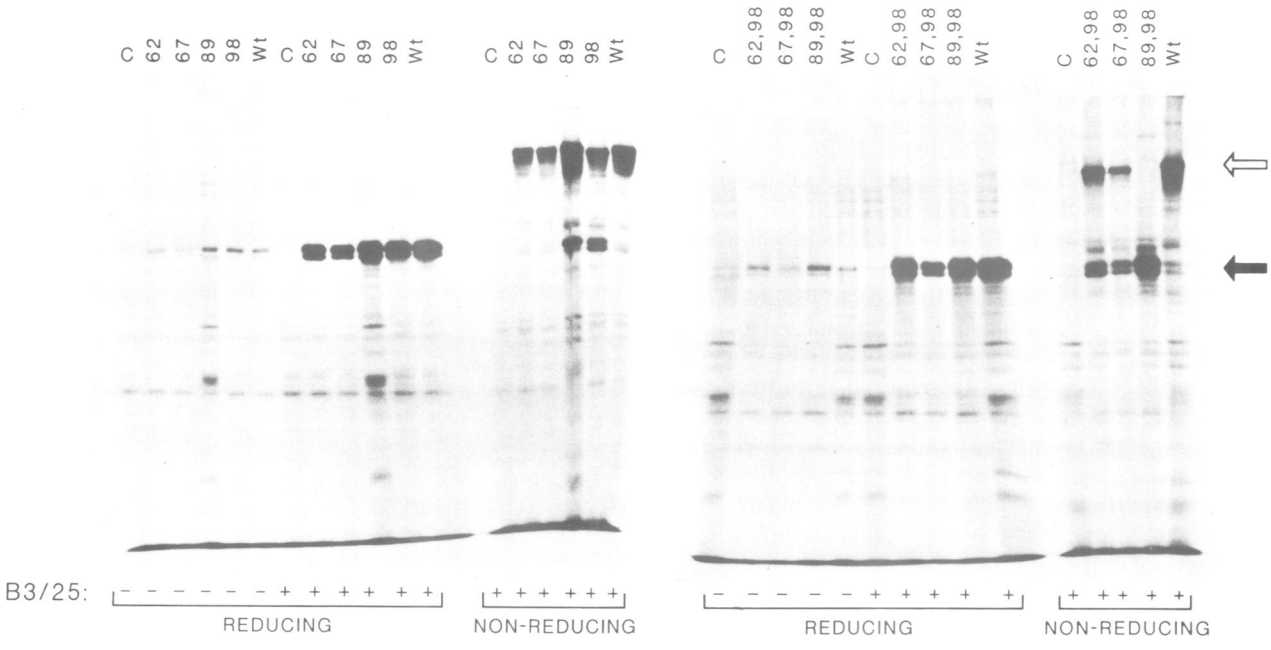

Fig. 3. Identification of the cysteine residues in the human transferrin receptor involved in intermolecular disulfide bonds. The figure shows fluorographs of the human transferrin receptor isolated by immunoprecipitation with B3/25 monoclonal antibody from lysates of Cos cells transfected with JC119 [control (C)], pJTR [encoding the wild-type receptor (Wt)] or mutant constructs and then metabolically labeled with [^{35}S]methionine. Samples were analyzed by SDS–polyacrylamide gel electrophoresis on 7.5% acrylamide gels. Fluorography of the gels was for 8 h. Further experimental details are given in Materials and methods. The **left-hand panel** shows the analysis of single mutants in which one cysteine has been converted to serine. The **right-hand panel** shows the analysis of double mutants in which cysteine 98 and either cysteine 62, 67 or 89 have been converted to serine.

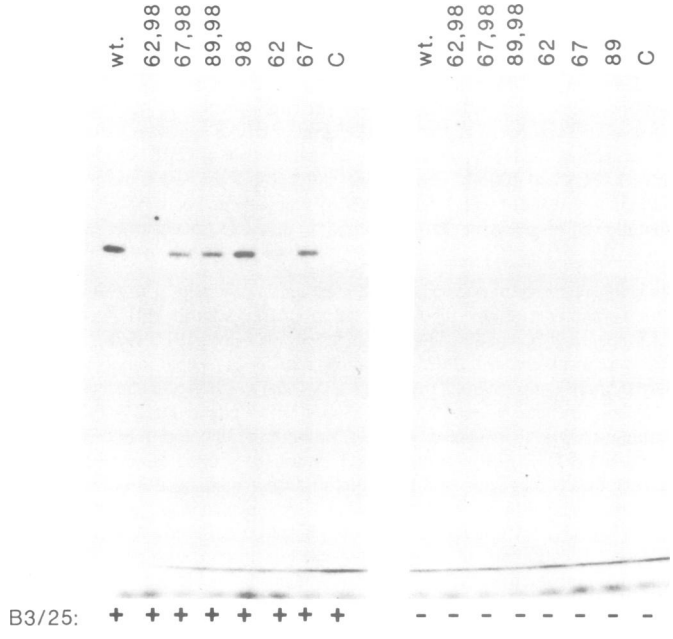

Fig. 4. Acylation of mutant transferrin receptors expressed in transfected Cos cells. Cos cells were transfected with pJTR, mutant constructions, or JC119 and then 40–48 h later labeled with [^{3}H]palmitate. Immunoprecipitates were prepared and analyzed by SDS–polyacrylamide gel electrophoresis. The fluorograph shown was exposed for 21 days. Additional experimental details are given in Materials and methods. Although the [^{3}H]palmitate labeling of the human transferrin receptor from Cos cells transfected with pJTR-Ser 89 is not included in this experiment, in other experiments the labeling was similar to that of pJTR-Ser 89, 98 shown here.

brane-spanning region. When the JC119 constructs containing the modified transferrin receptor cDNAs were expressed in Cos cells, it was found that the mutant receptors with two cysteines modified to serine were all transported to the cell surface (Figure 2). The results of metabolic labeling experiments with [^{35}S] methionine are shown in Figure 3, right-hand panel. It is clear that the double mutant in which cysteine 89 and cysteine 98 have been converted to serine migrates as a monomer under non-reducing conditions. We conclude therefore that the human transferrin receptor has two intermolecular disulfide bonds involving these two cysteine residues.

Site of acylation of the human transferrin receptor

Previous work had suggested that one of the four cysteines around the transmembrane spanning region was the site of acylation of the transferrin receptor. To address this question, the mutant human transferrin receptor cDNAs with cysteine to serine modifications were expressed in Cos cells which were then metabolically labeled with [^{3}H]palmitate. The results of this experiment are shown in Figure 4. It can be seen that all the mutant molecules except that in which cysteine 62 has been modified to serine incorporate similar amounts of [^{3}H]palmitate. In contrast, the cysteine to serine 62 mutant is not detectably labeled with palmitate even though it was shown in parallel metabolic labeling experiments with [^{35}S]methionine that similar amounts of receptor glycoprotein is synthesized and inserted in the cell membrane (see Figure 3). It is concluded, therefore, that the major site of lipid attachment in the human transferrin receptor is at cysteine 62.

Discussion

The results of oligonucleotide site-directed mutagenesis clearly establish that the human transferrin receptor contains two intermolecular disulfide bonds involving cysteine residues at position 89 and 98. Cysteine 98 is in the extracellular domain of the receptor and cysteine 89 at the junction between the extracellular domain and the transmembrane-spanning region. As modification of only one of the cysteines to serine did not convert the molecule

to monomeric form in denaturing, non-reducing conditions, it can be inferred that the disulfide linkages are between cysteine 89 from each subunit and cysteine 98 from each subunit. Both these cysteine residues are conserved in the mouse transferrin receptor (Stearne *et al.*, 1985). The mouse receptor is also dimeric (Trowbridge *et al.*, 1982) and it is likely that both homologous cysteines also form intermolecular disulfide bonds. In contrast, the chicken transferrin receptor, which has also been identified by means of a monoclonal antibody, has been shown to exist partially as a disulfide-bonded dimer and partially as a monomer which does not contain intermolecular disulfide bonds (Schmidt *et al.*, 1985). This is similar to the mutant human transferrin receptor molecules in which either cysteine 89 or 98 was converted to serine. It is possible, therefore, that in the chicken transferrin receptor only one of these cysteines is conserved. It is not known whether the mutant human transferrin receptor molecule which does not contain intermolecular disulfide bonds exists on the cell surface as a monomer or as a non-covalently associated dimer. Crosslinking studies using the bifunctional reagents, disuccinimidyl suberate (DSS), and dithiobis (succinimidyl propionate) (DSP) did not resolve this question. Over a concentration range of 0.05−0.5 mM of either reagent, both wild-type and mutant receptors lacking disulfide bonds displayed on transfected Cos cells were progressively crosslinked to high M_r complexes (>400 000) without detectable amounts of crosslinked dimer (results not shown). Crosslinking of the transferrin receptor on the human T leukemic cell line, CCRF-CEM, gave similar results. Whether this suggests that both wild-type and mutant transferrin receptors exist predominantly in multimeric clusters on the cell surface is unclear.

Site-directed mutagenesis has also been used to identify cysteine 62 as the major lipid attachment site. This residue is adjacent to the transmembrane spanning region of the receptor on the cytoplasmic face of the cell membrane. Although the murine transferrin receptor is also acylated (Sefton *et al.*, 1982), cysteine 62 in the human receptor is not conserved in the murine sequence and is replaced by phenylalanine (Stearne *et al.*, 1985). The results of the present experiments are not sufficiently quantitative (largely due to the variation in the fraction of Cos cells expressing the human transferrin receptor in individual transfection experiments) to exclude the possibility that in the human transferrin receptor, cysteine 67 is a minor acylation site. This residue is conserved in the murine receptor and is the likely site of acylation in this molecule. The attachment of palmitate to a cysteine residue close to or within the transmembrane-spanning region is consistent with similar observations that have been made for the transmembrane glycoprotein (G protein) of VSV (Rose *et al.*, 1984) and the HLA-B7 and HLA-DR heavy chains (Kaufman *et al.*, 1984).The modified transferrin receptors lacking the major acylation site and those without intermolecular disulfide bonds are transported to the cell surface and expressed. Neither structural feature is therefore essential for these processes. Further studies utilizing stable expression systems will be required to determine whether there are quantitative differences in the transport and expression of the mutant and wild-type receptors and whether their functional properties differ.

Materials and methods

Oligonucleotide site-directed mutagenesis

Oligonucleotide site-directed mutagenesis was carried out essentially as described by Rose *et al.* (1984). Oligonucleotides were made with a Microsyn 1450A Synthesizer using phosphoroamidate chemistry and purified by polyacrylamide gel electrophoresis (Rose *et al.*, 1984). Priming with the oligonucleotides and the conditions for the extension reaction were exactly as described (Rose *et al.*, 1984). A third of the primer-extension reaction product was used to transform competent JM101 or JM103 cells and the resulting infected colonies screened by differential hybridization to the 5′ $^{32}PO_4$-labeled oligonucleotide as described by Zoller and Smith (1983). Putative mutants were picked with toothpicks onto fresh agar plates and allowed to grow overnight and were then rescreened. Finally, single-stranded phage were prepared from positive colonies and used to infect JM101 or JM103 cells. Single infected colonies from this tertiary screen were then used to prepare mini-preps of the replicative form DNA. Each oligonucleotide was designed not only to convert cysteine to serine but also to introduce a new restriction site into the transferrin receptor cDNA (see Figure 1). Authentic plaque-purified mutants were thus identified by digesting the replicative form DNA from the mini-preps with the appropriate restriction enzyme. The human transferrin receptor 1.1-kb *Bam*HI−*Hin*dIII cDNA fragment containing the mutation isolated from M13mp19 together with the 1.9-kb *Hin*dIII−*Bgl*II cDNA fragment isolated from pcDTR-1 required to reconstruct the intact coding region of the human transferrin receptor (see Figure 1) were cloned into the *Bam*HI site of the JC119 expression vector (Sprague *et al.*, 1983; Rose *et al.*, 1984) using standard techniques (Maniatis *et al.*, 1982). Colonies of MC1061 cells transformed with DNA from this triple ligation reaction were screened for JC119 containing an insert of the mutant transferrin receptor cDNA first by hybridization to the ^{32}P-labeled 2.6-kb *Hin*dIII fragment of the receptor cDNA (Figure 1), then by differential hybridization to the appropriate 5′ $^{32}PO_4$-labeled oligonucleotide. Inserts in the correct orientation were identified by restriction mapping with *Pst*I. Finally, the mutations in the JC119 constructs used to transfect Cos cells were confirmed by sequencing the region of DNA containing the mutations using the method of Maxam and Gilbert (1977).

Transfection of plasmid DNA into Cos cells

The transfection of African green monkey Cos cells with the JC119 constructs was carried out as described (Rose and Bergmann, 1983; Rose *et al.*, 1984) with the following changes. For metabolic labeling experiments, monolayers of cells (~2.5 × 10^6 cells in 60-mm tissue culture dishes) were transfected with 15 μg of plasmid DNA in 2.0 ml of Tris−saline buffer containing 200 μg/ml of DEAE−Dextran. After 30−40 min incubation, the transfection buffer was replaced with 2.5 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and 100 μM chloroquine. After a further 3−4 h incubation, this medium was replaced with 3 ml of DMEM supplemented with 5% FCS. For immunofluorescence studies, transfections were carried out using a similar procedure. Monolayers of Cos cells were grown out on 22-mm sterilized glass coverslips and transfected in 30-mm petri dishes with 4 μg of plasmid DNA in 1.0 ml of Tris−saline containing 200 μg/ml of DEAE−dextran. After 30−40 min incubation, the DNA solution was replaced by 1 ml of DMEM supplemented with 5% FCS and 100 μM chloroquine. After a further 3−4 h incubation this medium was replaced with 1 ml of DMEM supplemented with 5% FCS and the cells allowed to grow for 40−48 h at 37°C in a CO_2 incubator.

Indirect immunofluorescence

Cos cells previously transfected 40−48 h earlier on coverslips with plasmid DNA were washed two to three times with 1.0 ml of Hepes buffer (pH 7) containing 0.2% polyvinyl pyrrolidinone and 5 mM NaN_3 (Lesley *et al.*, 1984). Then the cells were incubated with B3/25 anti-human transferrin receptor monoclonal antibody (Omary *et al.*, 1980; Trowbridge and Omary, 1981) at a concentration of 50 μg/ml in a total volume of 50 μl Hepes buffer containing 2% FCS. After washing, cells were incubated with a 1:75 dilution of fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse IgG (Miles Inc.) in the same buffer. After washing three times with 1 ml of Hepes buffer containing 0.2% polyvinyl pyrrolidinone and 5 mM NaN_3, the coverslips were mounted onto microscope slides using 10 μl of a 1:1 solution of glycerol−phosphate-buffered saline (PBS). The cells were then examined using a Nikon optiphot microscope equipped with fluorescent epi-illumination and a Nikon 40× oil immersion plan apochromat objective.

Metabolic labeling experiments

Metabolic labeling of transfected Cos cells with L-[^{35}S]methionine was carried out 40−48 h after transfection. Cells were metabolically labeled for 4 h at 37°C in 2.5 ml of methionine-free DMEM containing 50 μCi/ml L-[^{35} S]methionine (New England Nuclear, 1142 Ci/mM) and 2% dialyzed FCS. Metabolic labeling with [^{3}H]palmitic acid was also carried out 40−48 h after transfection in 2.5 ml of DMEM supplemented with 50 μCi/ml [^{3}H]palmitic acid (Amersham, 46 Ci/mM) and 2% FCS for 6 h at 37°C. The cells from each 60-mm dish were then washed with PBS and solubilized in 0.5 ml of 1% Nonidet P-40 (NP-40) in PBS containing 10 mM iodoacetamide. Cell lysates were pre-cleared using 100 μl of packed *Staphylococcus aureus* (Pansorbin, Calbiochem-Behringer). B3/25 monoclonal antibody (5 μg) was then added to each cell lysate. The antibody−antigen complexes were then bound to *S. aureus* pre-coated with rabbit anti-mouse IgG antibodies (Cooper Biomedical). The *S. aureus* with bound antibody−antigen complexes were then washed three times with 0.4 ml of 0.5% deoxycholate, 0.5% NP-40, 0.5% SDS in PBS. Samples were boiled for 2 min

in 50 μl of electrophoresis sample buffer (Maizel, 1971) with or without 2% mercaptoethanol. Aliquots (20 μl) were then analyzed by SDS–polyacrylamide gel electrophoresis on 7.5% acrylamide gels (Maizel, 1971). Following electrophoresis, gels were fixed and stained then processed for fluorography (Bonner and Laskey, 1974).

Chemical crosslinking of cell surface proteins

Transfected Cos cells or human T leukemic CCRF-CEM cells were labeled by cell surface iodination (Omary and Trowbridge, 1981a), treated with 25 μM desferrioxamine in PBS (pH 7.2) for 10 min at 23°C, and then crosslinked with either 0.05, 0.2 or 0.5 mM DSP or DSS in PBS (pH 8.0) for 30 min at 4°C. Immunoprecipitates were then prepared from solubilized cells and analyzed under reducing and non-reducing conditions as described for metabolically labeled cells.

Acknowledgements

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