A point mutation in the cytoplasmic domain of the transferrin receptor inhibits endocytosis

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A point mutation in the cytoplasmic domain of the transferrin receptor inhibits endocytosis

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The rate of receptor-mediated endocytosis of diferric $^{125}$I-transferrin by Chinese-hamster ovary cells expressing human transferrin receptors was compared with the rate measured for cells expressing hamster transferrin receptors. It was observed that the rate of endocytosis of the human transferrin receptor was significantly higher than that for the hamster receptor. In order to examine the molecular basis for the difference between the observed rates of endocytosis, a cDNA clone corresponding to the cytoplasmic domain of the hamster receptor was isolated. The predicted primary sequence of the cytoplasmic domain of the hamster transferrin receptor is identical with that of the human receptor, except at position 20, where a tyrosine residue in the human sequence is replaced with a cysteine residue. To test the hypothesis that this structural change in the receptor is related to the difference in the rate of internalization, we used site-directed mutagenesis to examine the effect of the replacement of tyrosine-20 with a cysteine residue in the human transferrin receptor. It was observed that the substitution of tyrosine-20 with cysteine caused a 60% inhibition of the rate of iron accumulation by cells incubated with $[^{59}$Fe]$^{2+}$diferric transferrin. No significant difference between the rate of internalization of the mutant (cysteine-20) human receptor and the hamster receptor was observed. Thus the substitution of tyrosine-20 with a cysteine residue can account for the difference between the rate of endocytosis of the human and hamster transferrin receptors.

INTRODUCTION

The cytoplasmic domain of the transferrin receptor has been demonstrated to be required for the clustering of the receptors in coated pits and subsequent endocytosis (Rothenberger et al., 1987; Iacopetta et al., 1988). A role for the cytoplasmic domain has also been established for the endocytosis of the polymeric Ig receptor (Mostov et al., 1986), the low-density-lipoprotein (LDL) receptor (Anderson et al., 1977; Davis et al., 1987a), and the cation-independent mannose 6-phosphate receptor (Lobel et al., 1989). Little is known about the structural information that is present in the cytoplasmic domains of these receptors that determines the sorting process during internalization. In the case of the LDL receptor, tyrosine-807 has been reported to play an important role in receptor-mediated endocytosis (Davis et al., 1987a). Recently it has been reported that tyrosine-24 and tyrosine-26 are required for the efficient endocytosis of the cation-independent mannose 6-phosphate receptor (Lobel et al., 1989). Furthermore, it has been demonstrated that the replacement of cysteine-543 with a tyrosine residue in the influenza haemagglutinin (HA) protein cytoplasmic tail allows this protein to assemble into coated pits and undergo endocytosis (Lazarovits & Roth, 1988), whereas the native protein is excluded from plasma-membrane coated pits (Roth et al., 1986). Together these studies suggest that a tyrosine residue in the cytoplasmic domain of receptors may be part of a structure that is required for clustering in coated pits.

The purpose of the experiments reported here was to obtain evidence for a specific region of the transferrin receptor cytoplasmic domain that is required for endocytosis. The approach that we took was to compare the structure and function of the human and hamster transferrin receptors.

EXPERIMENTAL

Materials

$^{125}$I, $^{35}$S-labelled dATP, [deoxyadenosine 5'-($^{35}$S)thio]triphosphate, [$^{32}$P]ATP and FeCl$_3$ were from Amersham International. Human transferrin was from Behring Diagnostics and was saturated with iron as described by Davis & Czech (1986). Diferric $^{125}$I-transferrin (5–7 Ci/g) and $[^{59}$Fe]$^{2+}$diferric transferrin (50 to 40 mCi/g) were prepared as described by Davis & 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. Sequenase and Thermus aquaticus DNA polymerase were obtained from United States Biochemical Corp. and Perkin Elmer Cetus respectively. All other enzymes were from Boehringer.

Tissue culture

CHO-K1 cells and 3T3 L1 pre-adipocytes were obtained from the American Type Culture Collection. The isolation of TF$^{-}$ cells has been previously described (Alvarez et al., 1989). 3T3 L1 pre-adipocytes were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% (v/v) calf serum. CHO and TF$^{-}$ cells were maintained in Ham's F12 medium supplemented with 5% (v/v) fetal-bovine serum (Gibco). Human transferrin receptors were expressed in TF$^{-}$ cells by transfection of the cells using the calcium phosphate method (Alvarez et al., 1989). Transfected cells were selected by incubation with modified Eagle’s medium supplemented with 500 nm-amethopterin, 0.25 $\mu$M-Fe(NO$_3$)$_3$ and 5% dialysed fetal-bovine serum. Stable colonies were obtained and were isolated by using cloning rings (Alvarez et al., 1989).

Abbreviations used: CHO, Chinese-hamster ovary; HA, haemagglutinin; LDL, low-density lipoprotein.
Polymerase chain reaction
The template used for the polymerase chain reaction was a hamster cDNA library (Chin et al., 1984) obtained from Dr. G. Gil (University of Massachusetts Medical School). The primers used were 5'-GTA GCC A/C(A)/T CAT (A/G) AA TCC AATT-CA A GAA-3' and 5'-CG CAT GTT CTT CTT CT GT T GG CAG- T T C-3'. The template (1 µg) was mixed with two primers (100 pmol) in 10 µM-Tris (pH 8.3)/50 mM-KCl/1.5 mM-MgCl₂/200 µM-dATP/200 µM-dCTP/200 µM-dGTP/200 µM-dTTP/0.01% (w/v) gelatin (final volume of 100 µl). The reaction mixture was heated to 100 °C for 10 min and cooled to 68 °C for 10 min. Thermus aquaticus DNA polymerase (2.5 units) was added during the incubation at 68 °C. In all, 35 cycles of amplification were then performed (denaturation, 1.5 min at 94 °C; annealing, 1.5 min at 50 °C; extension, 5 min at 68 °C). The amplified DNA was phenol-extracted, ethanol-precipitated, and analysed by polyacrylamide-gel electrophoresis. A 285 bp amplified fragment was observed. The 285 bp DNA fragment was eluted from the gel and cloned into M13mp18 at the restriction endonuclease SmaI site. Ten M13 clones were picked and sequenced using the dNTP chain-termination method (Sanger et al., 1977). The experiment was repeated twice. The sequence of the 285 bp fragment was the same for all the clones examined.

Plasmid construction
The human transferrin receptor cDNA (Kuhn et al., 1984; McClelland et al., 1984) was obtained from Dr. F. Ruddle (Yale University). A BamHI–HindIII 0.9 kb fragment of the cDNA (which contains the 5'-untranslated region and the coding region for the cytoplasmic domain) was subcloned into M13mp18 as described by Davis & Meisner (1987). Site-directed mutagenesis of tyrosine-20 was carried out as described by Zoller & Smith (1984) using 18-mer oligonucleotides coding for cysteine (5'-A A C C G G GT A C AT G A C A A T-3') and phenylalanine (5'-A A C C G G G T A A T G A C A A T-3'). Mutations were confirmed by sequencing using 32P-labelled dATP, ddNTPs and Sequenase (Sanger et al., 1977). Replicative-form DNA was isolated and the cDNA fragment prepared by endonuclease digestion and agarose-gel electrophoresis. This fragment was ligated to a HindIII–BglII 1.9 kb fragment of the receptor cDNA that contains the coding region for the C-terminal domain of the receptor, and the full-length cDNA was isolated by agarose-gel electrophoresis (Davis & Meisner, 1987). The full-length wild-type and mutant cDNAs were then cloned into an expression vector as described by Alvarez et al. (1989). This vector contains the mouse dihydrofolate reductase gene as a selectable marker and allows the expression of the transferrin receptor cDNA by utilizing the simian-virus-40 early promoter and polyadenylation signals. The correct orientation of the cDNAs cloned into the expression vector was determined by digestion with restriction endonucleases.

Binding of diferric 125I-transferrin to cell-surface receptors
Binding assays were performed on cells grown in 16 mm-diameter wells. The cells were washed with serum-free medium and incubated for 30 min at 37 °C in 30 µM bovine serum albumin/120 mM-NaCl/6 mM-KCl/1.2 mM-MgCl₂/1 mM-CaCl₂/10 mM-glucose/25 mM-Hepes, pH 7.4. The medium was replaced and rapidly replaced with medium at 4 °C. Diferric 125I-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 1M-NaOH. Radioactivity associated with the cells was quantified with a Beckman γ-radiation counter. Non-specific binding was estimated in incubations with a 200-fold excess of unlabelled ligand.

Transferrin-receptor endocytosis
The endocytotic rate constant for transferrin-receptor internalization was measured by the In/Sur method described by Wiley & 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 125I-transferrin. At defined times the binding of the 125I-transferrin to the cells was measured by rapidly washing the cell monolayers at 4 °C and determining the associated radioactivity with a γ-radiation counter. Cell-surface and intracellularly bound 125I-transferrin was determined by incubation of the cells for 3 min at 4 °C with 50 mM-NaCl/150 mM-glucose, pH 3.0. Intracellular 125I-transferrin was estimated by measurement of the cell-associated radioactivity after acid washing (Haigler et al., 1980; Lamb et al., 1983). Cell-surface-bound 125I-transferrin was estimated by subtraction of the intracellularly bound 125I-transferrin from the total specific binding of 125I-transferrin to cell monolayers. Non-specific binding of 125I-transferrin was estimated in incubations containing a 200-fold excess of unlabelled ligand.

Transferrin-receptor recycling
The apparent first-order rate constant for the recycling of transferrin receptors was estimated by measuring the rate of recycling of 125I-transferrin as described previously (Alvarez et al., 1989). CHO cells were seeded in 16 mm wells and grown to a density of 5 x 10⁴ cells per well. The cells were then incubated with 10 nM diferric 125I-transferrin for 2 h at 37 °C. The medium was removed and the cells were washed at 0 °C and subsequently incubated for 3 min at 0 °C with 50 mM-NaCl/150 mM-glucose, pH 3.0, to remove ligand bound to the cell surface. The acid-washing procedure caused the dissociation of more than 95% of 125I-transferrin specifically bound to cell-surface receptors (results not shown). To investigate the rate of recycling of 125I-transferrin, the cells were incubated with medium at 37 °C to initiate the release of intracellular 125I-transferrin for defined times. The radioactivity remaining associated with the cells was measured with a radio counter. The apparent first-order rate constant for the release of 125I-transferrin was estimated using the computer program ENZFITTER (Elsevier Biosoft). Previously, the rate of 125I-transferrin recycling has been determined in experiments employing a pulse-chase procedure without an acid wash (Davis et al., 1987b). The rates of recycling of 125I-transferrin measured in experiments performed without Davis et al., 1987b) and with Alvarez et al., 1989) an acid wash are similar. We therefore conclude that the acid-washing procedure employed in the present study does not cause a marked inhibition of the recycling of 125I-transferrin.

Accumulation of 15Fe-diferric transferrin
CHO cells were seeded in 16 mm wells and grown to a density of 5 x 10⁴ 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-MgCl₂/1 mM-CaCl₂/25 mM-Hepes, pH 7.4 containing 30 µM bovine serum albumin. The cells were then incubated for different times with 300 nM-[15Fe]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 by Davis et al (1986).

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RESULTS

Human transferrin receptors were expressed in a CHO-K1 variant (clone TF) that lacks endogenous transferrin receptors (Alvarez et al., 1989). The first-order rate constant for the endocytosis of the human receptor expressed in TF cells was measured to be 0.32 min⁻¹ (Table 1). This rate is similar to that previously reported for the endocytosis of the transferrin receptor in A431 human epidermoid-carcinoma cells, namely 0.33 min⁻¹ (Davis et al., 1987b). In contrast with the rapid endocytosis of the human transferrin receptor, the rate of endocytosis of the hamster transferrin receptor in CHO-K1 cells was found to be significantly slower (Table 1). These data indicate that the hamster transferrin receptor is internalized less efficiently than the human receptor.

To examine the molecular basis for the lower rate of endocytosis of the hamster transferrin receptor, a cDNA clone corresponding to the cytoplasmic domain of the receptor was isolated from a CHO-cell library. The sequence of the clone obtained was very similar to the published sequence of the human transferrin receptor (McClelland et al., 1984; Schneider et al., 1984). Only two differences between the cDNA sequence corresponding to the cytoplasmic domains of the human and hamster receptors were observed. The first difference was found at codon 8, where the human and hamster sequences were GCT (Ala) and GCA (Ala) respectively. The second difference was detected at codon 20, where TGT (Cys) was found in the hamster cDNA in contrast with TAT (Tyr) in the human sequence. The predicted amino acid sequence of the cytoplasmic domains of the human and hamster transferrin receptors are presented in Fig. 1.

There is a single amino acid difference between the primary sequence of the cytoplasmic domains of the human and hamster transferrin receptors at residue 20 (Fig. 1). As evidence has been reported indicating that the cytoplasmic domain of the receptor plays a role in endocytosis (Rothenberger et al., 1987; Iacopetta et al., 1988), it is possible that this difference in sequence accounts for the lower observed rate of internalization of the hamster transferrin receptor compared with the human receptor. To test this hypothesis, the effect of replacement of tyrosine-20 in the human transferrin receptor with a cysteine residue was examined using site-directed mutagenesis. In a control experiment the effect of the replacement of tyrosine-20 with a phenylalanine residue was investigated.

TF² cells expressing [Tyr²⁰], [Cys²⁰], or [Phε²⁰]-human transferrin receptors were isolated. Investigation of the binding isotherm demonstrated that the Kₐ (mean ± S.E.M.) for diferric ²⁰¹⁻transferrin was 0.88 ± 0.09 nM ([Tyr²⁰]) 0.73 ± 0.06 nM ([Cys²⁰]) and 0.81 ± 0.16 nM ([Phε²⁰]). These data indicate that the mutation of the cytoplasmic domain did not significantly alter the affinity of the receptor for diferric ²⁰¹⁻transferrin. The function of the wild-type and mutant transferrin receptors were characterized by examination of the rates of receptor endocytosis and recycling. Table 1 shows that the rate of recycling of the [Tyr²⁰], [Phε²⁰], and [Cys²⁰]-human transferrin receptors was not significantly different. However, the rate of endocytosis of the [Phε²⁰] and [Cys²⁰]-human transferrin receptors was significantly lower than that observed for the wild-type [Tyr²⁰] receptor (Table 1). Thus the substitution of tyrosine-20 with a cysteine or a phenylalanine residue caused a diminution in the rate of endocytosis without altering the rate of receptor recycling (Table 1). These changes in the kinetic parameters for receptor cycling were associated with an increase in the expression of the mutant receptors at the cell surface compared with the wild-type receptor (Table 2). To examine the significance of these changes in receptor cycling, the accumulation of iron by cells incubated with [⁵⁹Fe]diferric transferrin was investigated. Fig. 2 shows that the rate of iron uptake by the [Cys²⁰]- and [Phε²⁰]-human

Table 1. Summary of kinetic rate constants for transferrin-receptor recycling

<table>
<thead>
<tr>
<th>Species...</th>
<th>Hamster [Cys²⁰]</th>
<th>Human [Tyr²⁰]</th>
<th>Human [Phε²⁰]</th>
<th>Human [Cys²⁰]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocytosis</td>
<td>0.24 ± 0.014</td>
<td>0.32 ± 0.023</td>
<td>0.25 ± 0.015</td>
<td>0.23 ± 0.029</td>
</tr>
<tr>
<td>Recycling</td>
<td>0.137 ± 0.005</td>
<td>0.085 ± 0.003</td>
<td>0.10 ± 0.024</td>
<td>0.11 ± 0.016</td>
</tr>
</tbody>
</table>

Table 2. Cell-surface expression of [Tyr²⁰], [Phε²⁰], and [Cys²⁰]-human transferrin receptors

<table>
<thead>
<tr>
<th>Receptor...</th>
<th>Wild-type [Tyr²⁰]</th>
<th>[Phε²⁰]</th>
<th>[Cys²⁰]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellsurface</td>
<td>21.3 ± 2.1</td>
<td>30.7 ± 2.0</td>
<td>37.7 ± 1.1</td>
</tr>
<tr>
<td>Intracellular</td>
<td>78.7 ± 2.1</td>
<td>69.3 ± 2.0</td>
<td>62.3 ± 1.0</td>
</tr>
</tbody>
</table>
transferrin receptors was 38 and 62% of the rate measured for the wild-type [Tyr80]receptor respectively.

DISCUSSION

It has been demonstrated that the cytoplasmic domain of the transferrin receptor is required for the clustering of the receptor into plasma-membrane coated pits and subsequent endocytosis. As an initial approach to investigate whether a specific region of the cytoplasmic domain is required for endocytosis, we have compared the structure and function of the human and hamster transferrin receptors. It was observed that the rate of endocytosis of the human receptor expressed in CHO cells was similar to the rate measured in A431 human epidermoid-carcinoma cells. By contrast, the rate of endocytosis of the hamster receptor in CHO cells was significantly lower than that of the human receptor. To investigate the molecular basis for the low rate of endocytosis of the hamster receptor, a cDNA clone corresponding to the cytoplasmic domain of the hamster receptor was isolated from a CHO-cell library. A single amino acid difference was found between the predicted primary sequence of the cytoplasmic domains of the human and hamster transferrin receptors (Fig. 1). Residue 20 was tyrosine and cysteine in the human and hamster receptors respectively. To test the hypothesis that this structural change in the receptor is related to the difference in the rate of internalization we used site-directed mutagenesis to examine the effect of the replacement of tyrosine-20 with a cysteine residue in the human transferrin receptor. The data obtained demonstrate that the substitution of tyrosine-20 with a cysteine residue can account for the difference between the rate of endocytosis of the human and hamster transferrin receptors (Table 1).

Previous studies have indicated a role for a tyrosine residue in the cytoplasmic domain of the LDL receptor in the process of endocytosis (Davis et al., 1987a). Substitution of tyrosine-807 in the LDL receptor with a cysteine residue caused a 78% inhibition of the internalization of 125I-LDL (Davis et al., 1987a). In the present study, substitution of transferrin-receptor tyrosine-20 with a cysteine residue caused a 62% inhibition of the internalization of [55Fe]diferric transferrin (Fig. 2). These data indicate that the substitution of the tyrosine residue in the cytoplasmic domains of these receptors with cysteine caused qualitatively similar effects on endocytosis. By contrast, distinct effects of substitution with phenylalanine were observed for the LDL and transferrin receptors. Although the replacement of LDL-receptor tyrosine-807 with phenylalanine had no significant effect on endocytosis (Davis et al., 1987a), the substitution of transferrin-receptor tyrosine-20 with phenylalanine caused an inhibition of the internalization of diferric transferrin (Fig. 2). Studies with the influenza HA protein indicate that replacement of cysteine-543 with a tyrosine residue in the cytoplasmic tail allows this protein to assemble into coated pits and undergo endocytosis (Lazarovits & Roth, 1988), whereas the native protein is excluded from plasma-membrane coated pits (Roth et al., 1986). Substitution of cysteine-543 with phenylalanine did not allow the HA protein to rapidly internalize (Lazarovits & Roth, 1988). The effect of replacement of tyrosine with cysteine or phenylalanine in the recombinant HA protein is therefore more similar to the observations made with the transferrin receptor (Fig. 2) than with the LDL receptor (Davis et al., 1987a). The difference observed for the effects of substitution of tyrosine residues with cysteine and phenylalanine between the LDL receptor and the transferrin receptor suggests that tyrosine forms only a part of a recognition domain that is required for endocytosis. Consistent with this hypothesis is the finding that the location of the tyrosine residue in the short cytoplasmic tail of the HA protein is critical for rapid endocytosis (Lazarovits & Roth, 1988).

It has been previously demonstrated that the protein kinase C phosphorylation site on the transferrin receptor, namely serine-24 (Davis et al., 1986), is not required for receptor-mediated endocytosis of diferric transferrin (Davis & Meisner, 1987; Rothengerber et al., 1987; Zerial et al., 1987; McGraw et al., 1988). The present study indicates a role for tyrosine-20 during transferrin-receptor endocytosis. It is possible that tyrosine-20 forms part of a recognition domain that allows transferrin-receptor endocytosis, but further work is required to identify this structure. A goal for future studies will be to define the function of receptor structures that are required for endocytosis. Recently, the binding of the cytoplasmic domains of receptors to the HA-II adaptors of plasma-membrane coated vesicles has been reported (Pears, 1988; Glickman et al., 1989). It is possible that this binding of receptors to HA-II adaptors may represent an initial step in the pathway of endocytosis.

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REFERENCES

Role of transferrin receptor tyrosine-20

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