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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]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-liproprotein (LDL) receptor (Anderson *et al.*, 1977; Davis *et al.*, 1987*a*), 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.*, 1987*a*). 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

Na^{125}I, ^{35}S-labelled dATP, [deoxyadenosine 5′-(α-[^{35}S]thio)-triphosphate], [γ-^{32}P]ATP and $^{59}FeCl_3$ 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]diferric transferrin (30–40 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 α supplemented with 500 nM-amethopterin, 0.25 μM-$Fe(NO_3)_3$ 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′-GTAGCCA(C/A)TCAT(A/G)AATCCAATCAAGAA-3′and5′-CGCTAGTGTTCTTCTGTGTGGCAGTTC-3′. The template (1 μg) was mixed with the two primers (100 pmol) in 10 mM-Tris (pH 8.3)/50 mM-KCl/1.5 mM-$MgCl_2$/200 μM-dATP/200 μM-dCTP/200 μM-dTTP/200 μM-dGTP/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 *Sma*I site. Ten M13 clones were picked and sequenced using the ddNTP 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 *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 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′-AACCGGGTACATGACAAT-3′) and phenylalanine (5′-AACCGGGATAATGACAAT-3′). Mutations were confirmed by sequencing using ^{35}S-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 *Hin*dIII–*Bgl*II 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 ^{125}I-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-$MgCl_2$/1 mM-$CaCl_2$/10 mM-glucose/25 mM-Hepes, pH 7.4. 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 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 ^{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 γ-radiation 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 measurement 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 to cell monolayers. Non-specific binding of ^{125}I-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 ^{125}I-transferrin as described previously (Alvarez *et al.*, 1989). 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 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). Control experiments demonstrated that the acid-washing procedure caused the dissociation of more than 95% of ^{125}I-transferrin specifically bound to cell-surface receptors (results not shown). To investigate the rate of recycling of ^{125}I-transferrin, the cells were incubated with medium at 37 °C to initiate the release of intracellular ^{125}I-transferrin for defined times. The radioactivity remaining associated with the cells was measured with a radiation counter. The apparent first-order rate constant for the release of ^{125}I-transferrin was estimated by using the computer program ENZFITTER (Elsevier Biosoft). Previously, the rate of ^{125}I-transferrin recycling has been determined in experiments employing a pulse–chase procedure without an acid wash (Davis *et al.*, 1987*b*). The rates of recycling of ^{125}I-transferrin measured in experiments performed without (Davis *et al.*,1987*b*) 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 ^{125}I-transferrin.

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 containing 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 by Davis & Czech (1986).

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.*, 1987*b*). 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.

TF$^-$ cells expressing [Tyr^{20}]-, [Cys^{20}]- or [Phe^{20}]-human transferrin receptors were isolated. Investigation of the binding isotherm demonstrated that the K_d (mean ± S.E.M.) for diferric ^{125}I-transferrin was 0.88 ± 0.09 nM ([Tyr^{20}]-) 0.73 ± 0.06 nM ([Cys^{20}]-) and 0.81 ± 0.16 nM ([Phe^{20}]-). These data indicate that the mutation of the cytoplasmic domain did not significantly alter the affinity of the receptor for diferric ^{125}I-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 [Tyr^{20}]-, [Phe^{20}]- and [Cys^{20}]-human transferrin receptors were not significantly different. However, the rate of endocytosis of the [Phe^{20}]- and [Cys^{20}]-human transferrin receptors was significantly lower than that observed for the wild-type [Tyr^{20}]-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 [^{59}Fe]diferric transferrin was investigated. Fig. 2 shows that the rate of iron uptake by the [Cys^{20}]- and [Phe^{20}]-human

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                1                                                  13
Human     NH2 Met Met Asp Gln Ala Arg Ser Ala Phe Ser Asn Leu Phe
Mouse     NH2  -   -   -   -   -   -   -   -   -   -   -   -   -
Hamster   NH2  -   -   -   -   -   -   -   -   -   -   -   -   -

               14                                                  27
Human     Gly Gly Glu Pro Leu Ser Tyr Thr Arg Phe Ser Leu Ala Arg
Mouse      -   -   -   -   -   -   -   -   -   -   -   -   -   -
Hamster    -   -   -   -   -   -  Cys  -   -   -   -   -   -   -

               28                                                  41
Human     Gln Val Asp Gly Asp Asn Ser His Val Glu Met Lys Leu Ala
Mouse      -   -   -   -   -   -   -   -   -   -   -   -   -   -
Hamster    -   -   -   -   -   -   -   -   -   -   -   -   -   -

               42                                                  55
Human     Val Asp Glu Glu Glu Asn Ala Asp Asn Asn Thr Lys Ala Asn
Mouse     Ala  -   -   -   -   -   -   -   -   -  Met  -   -  Ser
Hamster    -   -   -   -   -   -   -   -   -   -   -   -   -   -

               56                  61
Human     Val Thr Lys Pro Lys Arg .....
Mouse      -  Arg  -   -   -   -
Hamster    -   -   -   -   -   -
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Fig. 1. Comparison of the primary sequence of the cytoplasmic domains of the human, mouse and hamster transferrin receptors

The predicted primary sequence of the cytoplasmic domains of the human, mouse and hamster transferrin receptors are presented. The human and mouse receptor sequences were taken from Schneider *et al.* (1984), McClelland *et al.* (1984), Stearne *et al.* (1985) and Rothenberger *et al.* (1987). The hamster receptor sequence was deduced from a partial cDNA clone that was obtained using polymerase-chain-reaction amplification of a hamster cDNA library (see the Experimental section).

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

The first-order rate constants for the endocytosis and recycling of the transferrin receptor were estimated from the rates of internalization of diferric ^{125}I-transferrin and release of ^{125}I-apotransferrin respectively. Results are means ± S.D. for three separate experiments.

	Rate constant (min^{-1})			
Species...	Hamster	Human	Human	Human
Receptor...	[Cys^{20}]-	[Tyr^{20}]-	[Phe^{20}]-	[Cys^{20}]-
Endocytosis	0.24 ± 0.014	0.32 ± 0.023	0.25 ± 0.015	0.23 ± 0.029
Recycling	0.137 ± 0.005	0.085 ± 0.003	0.10 ± 0.024	0.11 ± 0.016

Table 2. Cell-surface expression of [Tyr^{20}]-, [Phe^{20}]- and [Cys^{20}]-human transferrin receptors

TF$^-$ cells expressing [Tyr^{20}]-, [Phe^{20}]- and [Cys^{20}]-human transferrin receptors were incubated at 37 °C for 60 min with 10 nM-[^{125}I]diferric transferrin. The specific binding of ^{125}I-transferrin to cell-surface and intracellular receptors was measured as described in the Experimental section. Results are means ± S.D. for three separate experiments.

	Expression (%)		
		Mutant	
Receptor...	Wild-type [Tyr^{20}]-	[Phe^{20}]-	[Cys^{20}]-
Cell surface	21.3 ± 2.1	30.7 ± 2.0	37.7 ± 1.1
Intracellular	78.7 ± 2.1	69.3 ± 2.0	62.3 ± 1.0

transferrin receptors was 38 and 62% of the rate measured for the wild-type [Tyr^{20}]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.*, 1987*a*). Substitution of tyrosine-807 in the LDL receptor with a cysteine residue caused a 78% inhibition of the internalization of ^{125}I-LDL (Davis *et al.*, 1987*a*). In the present study, substitution of transferrin-receptor tyrosine-20 with a cysteine residue caused a 62% inhibition of the internalization of [^{59}Fe]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.*, 1987*a*), 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.*, 1987*a*). 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; Rothenberger *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 (Pearse, 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.

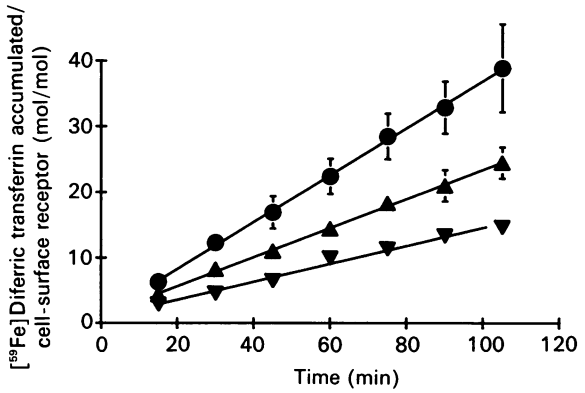

Fig. 2. Accumulation of [^{59}Fe]diferric transferrin

TF$^-$ cells expressing [Tyr^{20}]- (●), [Phe^{20}]- (▲), and [Cys^{20}]- (▼) human transferrin receptors were incubated for different periods with 300 nM-[^{59}Fe]diferric transferrin. The cells were then washed and the radioactivity associated with the cells was measured by 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 using the computer program LIGAND (Munson & Rodbard, 1980). The results are presented as mol of [^{59}Fe]diferric transferrin accumulated per mol of cell-surface receptors. Results are means ± S.D. for three separate experiments.

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