Role of the Human Transferrin Receptor Cytoplasmic Domain in Endocytosis: Localization Of a Specific Signal Sequence for Internalization

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Abstract. Wild-type and mutant human transferrin receptors have been expressed in chicken embryo fibroblasts using a helper-independent retroviral vector. The internalization of mutant human transferrin receptors, in which all but four of the 61 amino acids of the cytoplasmic domain had been deleted, was greatly impaired. However, when expressed at high levels, such "tailless" mutant receptors could provide chicken embryo fibroblasts with sufficient iron from diferric human transferrin to support a normal rate of growth. As the rate of recycling of the mutant receptors was not significantly different from wild-type receptors, an estimate of relative internalization rates could be obtained from the distribution of receptors inside the cell and on the cell surface under steady-state conditions. This analysis and the results of iron uptake studies both indicate that the efficiency of internalization of tailless mutant receptors is ∼10% that of wild-type

receptors. Further studies of a series of mutant receptors with different regions of the cytoplasmic domain deleted suggested that residues within a 10–amino acid region (amino acids 19–28) of the human transferrin receptor cytoplasmic domain are required for efficient endocytosis. Insertion of this region into the cytoplasmic domain of the tailless mutant receptors restored high efficiency endocytosis. The only tyrosine residue (Tyr 20) in the cytoplasmic domain of the human transferrin receptor is found within this 10–amino acid region. A mutant receptor containing glycine instead of tyrosine at position 20 was estimated to be ∼20% as active as the wild-type receptor. We conclude that the cytoplasmic domain of the transferrin receptor contains a specific signal sequence located within amino acid residues 19–28 that determines high efficiency endocytosis. Further, Tyr 20 is an important element of that sequence.

THE transferrin receptor (TR)[1] binds diferric transferrin (Tf) and mediates uptake of iron into the cell (reviewed in Trowbridge and Shackelford, 1985). It is a member of the class of transport receptors that are selectively concentrated in coated pits on the cell surface, constitutively internalized, and then recycled back to the cell surface (Goldstein et al., 1985). After endocytosis via coated pits (Hopkins and Trowbridge, 1983), TR and its bound ligand, diferric Tf, are routed into a mildly acidic endosomal compartment in which iron dissociates from Tf (van Renswoude et al., 1982; Dautry-Varsat et al., 1983). The TR-apoTf complex is then recycled back to the cell surface, whereupon apoTf dissociates, allowing the receptor to undergo further rounds of iron uptake. In most cells, TR-mediated iron transport is tightly coupled to cell growth (Trowbridge, 1985). The human TR is a homodimer of two 95-kD subunits covalently linked by two disulfide bonds

(Jing and Trowbridge, 1987). It has an external domain of 671 amino acids, a transmembrane region of 28 amino acids, and an amino-terminal cytoplasmic domain of 61 amino acids (Schneider et al., 1984; McClelland et al., 1984).

Previous studies of the receptors for low density lipoprotein (LDL) (Lehrman et al., 1985), poly-Ig (Mostov et al., 1986), mannose-6-phosphate (Lobel et al., 1989), and IgG (Miettinen et al., 1989) have demonstrated that their cytoplasmic domains contain structural determinants required for rapid internalization. Mutations in the 50-amino acid cytoplasmic domain of the LDL receptor that occur naturally in patients with familial hypercholesterolemia markedly inhibit clustering of receptors in coated pits and prevent high efficiency endocytosis (Anderson et al., 1977; Davis et al., 1986; Brown and Goldstein, 1986). The first 22 amino acids of the cytoplasmic domain of the LDL receptor juxtaposed to the plasma membrane are sufficient for rapid internalization (Davis et al., 1987). Of these amino acids, Tyr 807 located 18 residues from the transmembrane region appears to be especially critical for high efficiency endocytosis (Davis

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1. *Abbreviations used in this paper:* apoTf, apotransferrin; CEF, chicken embryo fibroblasts; LDL, low density lipoprotein; Tf, transferrin; TR, transferrin receptor.

et al., 1987). Recently, evidence has been obtained that tyrosine residues in the cytoplasmic domains of the cation-independent mannose-6-phosphate receptor (Lobel et al., 1989) and a mutant influenza virus hemagglutinin (Lazarovits and Roth, 1988) are important for the internalization of these proteins.

The cytoplasmic domain of the TR has also been implicated in the high efficiency internalization of Tf. It was concluded from studies in which mutant human TRs were expressed at high levels in mouse Ltk⁻cells that a deletion of 36 amino acids in the cytoplasmic domain abrogated endocytosis and clustering in coated pits (Rothenberger et al., 1987; Iacopetta et al., 1988). In this report, we have investigated the role of the cytoplasmic domain of the human TR in receptor-mediated endocytosis in another experimental system. We have expressed wild-type and mutant human receptors in chicken embryo fibroblasts (CEF) using a helper-independent retroviral vector (Hughes et al., 1987). Because chicken TRs do not bind human Tf (Shimo-Oka et al., 1986), internalization of human Tf by recombinant human TRs expressed in CEF can be determined independently of endogenous wild-type chicken receptors. We demonstrate that internalization of both mutant human TRs, with the identical 36–amino acid deletion to that studied by Rothenberger et al. (1987), and essentially "tailless" mutant receptors, lacking all but 4 of the 61 amino acids of the cytoplasmic domain, is highly impaired but still detectable in CEF. By the analysis of a series of deletion mutants, we obtained evidence that a 10–amino acid region within the cytoplasmic domain of the human TR contains a specific signal sequence determining high efficiency endocytosis. Introduction of this sequence into the tailless mutant receptors restores rapid internalization and their ability to mediate iron uptake. Thus, this region of the cytoplasmic tail of the TR is both necessary and sufficient for rapid internalization of diferric Tf and a normal rate of iron uptake. The only tyrosine residue in the cytoplasmic domain of the human TR is located within this stretch of amino acids, and we show that it is an important element of the internalization signal.

Materials and Methods

Oligonucleotide Site-directed Mutagenesis

FnuD II–Xba I fragments containing the coding region of the human TR were excised from the vectors pJTR and pJTR-Ser 89,98 that encode the wild-type TR and a mutant TR in which Cys 89 and Cys 98 have been converted to serine residues (Jing and Trowbridge, 1987). The recessed Xba I 3′ ends were filled in using the Klenow fragment of *Escherichia coli* DNA polymerase and then cloned into the Sma I site of the adaptor plasmid CLA 12 (Hughes et al., 1987). Cla I fragments encoding the mutant and wild-type human TRs were then excised and cloned into the retroviral expression vector RCAS (Hughes et al., 1987). In later experiments, a related vector, BH-RCAS (Hughes, S. H., unpublished results) was also used. For mutagenesis, Cla I–BamH I adaptors were added to ends of the Cla I fragments, which were then cloned into the BamH I site of M13mp 19. Subsequently, the Cla I fragment encoding the wild-type human TR was cloned directly into the Cla I site of the phagemid pBluescript SK (Stratagene, La Jolla, CA). Oligonucleotides were prepared on a DNA synthesizer (8750; Biosearch, San Rafael, CA) using phosphoamidite chemistry and purified by PAGE. Oligonucleotide site-directed mutagenesis was performed with phage and, subsequently, phagemid templates by the method of Kunkel (1985) using the Mutagene mutagenesis kit (Bio-Rad Laboratories, Richmond, CA). Mutants were selected by restriction mapping or differential hybridization, and Cla I fragments encoding the mutant receptors were cloned into the RCAS vector. The mutations were verified by dideoxynucleotide se-

quencing (Sanger et al., 1977; Tabor and Richardson, 1987) of the RCAS constructs using the Sequenase kit (United States Biochemical Corp., Cleveland, OH) according to the manufacturer's directions. Immunoprecipitation studies of the human TRs with deletions in their cytoplasmic domain from cells metabolically labeled with [³⁵S]methionine were performed to verify that a molecule of the appropriate relative molecular mass was expressed (Jing and Trowbridge, 1987).

Cell Culture and Expression of Human TRs in CEF

CEF were prepared from fertilized eggs (SPAFAS Inc., Norwich, CT) and grown in DME supplemented with 1% (vol/vol) chicken serum, 1% (vol/vol) defined calf bovine serum (HyClone Laboratories, Logan, UT), 2% (vol/vol) tryptose phosphate broth (Difco Laboratories Inc., Detroit, MI). CEF were transfected with 30 μg retroviral construct DNA/10-cm tissue culture plate of ~40% confluent cells using the polybrene-DMSO method (Kawai and Nishizawa, 1984). Cultures were monitored for the expression of human TRs by indirect immunofluorescence staining of cells plated on coverslips with B3/25 mAb (Trowbridge and Omary, 1981). Virtually all cells expressed human TRs 2 wk after transfection and were usually used for experiments within the next 6 wk. For growth studies, cells were plated at a cell density of 1.5 × 10⁴ cells/cm² in 6-well Costar (Cambridge, MA) cluster dishes containing 3 ml of DME supplemented with 3% (vol/vol) horse serum, 2% tryptose phosphate broth, and 50 μg/ml diferric human Tf (Miles Scientific Div., Naperville, IL). Cells were removed each day from triplicate dishes with 0.05% trypsin in versene buffer and counted in a counter (Coulter Electronics Inc., Hialeah, FL). Cells selected for growth in DME supplemented with horse serum and human diferric Tf were routinely maintained under the same conditions.

¹²⁵I-labeled Tf Binding and Endocytosis

Diferric human Tf was labeled with ¹²⁵I to a specific activity of 2–4 μCi/μg using Enzymobeads (Bio-Rad Laboratories) according to the manufacturer's directions. The number of cell surface TRs and their dissociation constant were estimated by Scatchard analysis of ¹²⁵I-labeled Tf binding at 4°C. Cells were plated at a density of 5–7 × 10⁴ cells/cm² in 24-well Costar tissue culture plates 24 h before the binding assay. Cells were incubated in serum-free DME for 1 h at 37°C and then washed once with ice-cold 0.15 M NaCl, 0.01 M Na phosphate buffer (pH 7.4) containing 0.1% BSA (BSA-PBS). ¹²⁵I-labeled Tf (5–500 ng) in 0.15 ml BSA-PBS was added to duplicate wells and incubated at 4°C for 90 min. Cells were then washed three times with 0.5 ml ice-cold BSA-PBS and removed from the wells with 0.5 ml 1 M NaOH, and the radioactivity was counted in a gamma counter. Nonspecific binding at each concentration of ¹²⁵I-labeled Tf determined by addition of a 100-fold excess of unlabeled human Tf was <10% of specific binding. Cells in replicate wells were counted in a counter (Coulter Electronics Inc.).

For studies of the endocytosis of mutant and wild-type human TRs expressed on CEF, cells were prepared in triplicate wells as described for the binding studies. To measure the kinetics of internalization, cells were incubated with 4 μg/ml ¹²⁵I-labeled Tf on ice for 1 h and washed three times with 1 ml of ice-cold BSA-PBS. Prewarmed DME (0.5 ml) containing 0.1% BSA and 50 μg/ml unlabeled human Tf was then added to all wells except the zero time point, and cells were incubated at 37°C for various times. For the zero time point, ice-cold medium was added to the wells and removed immediately. After incubation at 37°C, the medium was transferred to tubes for counting, and the cells were washed three times with 1 ml BSA-PBS and then incubated twice for 3 min with 0.5 ml of 0.2 M acetic acid, 0.5 M NaCl (pH 2.4) to remove surface-bound ¹²⁵I-labeled Tf (Hopkins and Trowbridge, 1983). Cells were then removed from the wells with 1 M NaOH, and radioactivity in the medium, the acid wash, and the cell pellet was determined. More prolonged incubation with the acid wash did not effect the radioactivity released.

The distribution of human TRs on the cell surface and inside the cell at steady state was determined by incubating cells, plated out and preincubated as described for binding studies, with 4 μg/ml ¹²⁵I-labeled Tf at 37°C for 15, 30, and 60 min. After incubation for these periods, the acid wash procedure described for the kinetic experiments was used to distinguish surface-bound and internalized ¹²⁵I-labeled Tf.

Measurement of Receptor Recycling Rates

The recycling rates of mutant and wild-type receptors were determined as described by Tanner and Lienhard (1987). Cells were plated at a density of 5–7 × 10⁴ cells/cm² in 24-well Costar tissue culture plates 24 h before the

assay. Cells were preincubated in serum-free DME as described for the binding studies and then incubated with 4 μg/ml ^{125}I-labeled Tf at 37°C for 60 min. Cells were washed three times with 0.5 ml ice-cold BSA-PBS, and surface-bound Tf was removed by incubation in 1 ml of 150 mM NaCl, 2 mM $CaCl_2$ 20 mM Na acetate buffer (pH 5.0) containing 50 μM deferoxamine mesylate for 15 min at 4°C. This was followed by one wash with 0.5 ml PBS and further incubation for 20 min at 4°C with PBS containing 50 μM deferoxamine mesylate and 125 nM human apoTf. Cells were then washed three times with 0.5 ml ice-cold BSA-PBS, and triplicate cultures were incubated at 37°C for various times with 0.5 ml prewarmed DME containing 0.1% BSA and 50 μg/ml unlabeled human Tf. Radioactivity released into the medium and remaining associated with the cells was then determined. A fraction of the internalized ^{125}I-labeled Tf, which in these experiments ranged from 18–23%, was not released from the cells after incubation at 37°C for 1 h and was subtracted from the values of cell-associated radioactivity at other time points (Tanner and Lienhard, 1987). In each experiment, a set of cultures were treated with 0.5 ml of 0.2 M acetic acid, 0.5 M NaCl (pH 2.4) to confirm that the deferoxamine treatment completely removed surface-bound Tf. The rate of recycling of wild-type receptors could also be estimated by saturating surface TRs with ^{125}I-labeled Tf at 4°C and then measuring the increase in cell-associated radioactivity when cells were incubated at 37°C in the continued presence of saturating amounts of ^{125}I-labeled Tf. This alternative method gave results consistent with those obtained using the procedure of Tanner and Lienhard (1987).

[59]Fe Uptake Measurements

Human apoTf was labeled with [59]Fe ($FeCl_3$; Amersham Corp., Arlington Heights, IL) to a specific activity of 5–10 μCi/mg using nitrilotriacetate (Bates and Schlabach, 1973). Cells were plated at a density of 5–7 × 10^4 cells/cm^2 in 24-well Costar tissue culture plates 24 h before the assay. The following day, cells were washed twice in prewarmed serum-free DME and then were incubated in serum-free DME containing 20 μg/ml ^{59}Fe-Tf at 37°C for various times. After washing, cells from triplicate wells for each time point were removed in 0.5 ml 1 M NaOH, and radioactivity was counted in a gamma counter. The relative levels of human TRs expressed on the various CEF populations were determined in each experiment. After preincubation for 1 h at 37°C in serum-free DME, triplicate wells of cells were incubated with 4 μg/ml ^{125}I-labeled Tf on ice for 1 h and then washed three times with 1 ml of ice-cold BSA-PBS, and the radioactivity bound to the cells was determined.

Immunohistochemistry

CEF and CEF expressing wild-type or mutant human TRs were plated onto glass coverslips and 24 h later incubated with human Tf. The attached cells were then either fixed with 3% formaldehyde directly or incubated for 30 min at 37°C before being fixed. After fixation, they were quenched with glycine and incubated with gold complexes as described previously (Hopkins and Trowbridge, 1983). Tf-horseradish peroxidase was prepared as described previously (Hopkins, 1983). Cells were incubated with the conjugate for 30 min at 5°C, rinsed at 5°C, warmed at 37°C for 10 min, and then fixed and processed for electron microscopy as described in detail by Hopkins (1983).

Electron micrographs were taken on a transmission electron microscope (CM12; Philips Electronic Instruments, Inc., Mahwah, NJ), and quantitation of particles was performed directly on the screen.

Results

Expression of Wild-type and Mutant Human TRs on CEF

Efficient expression of wild-type and mutant human TRs in CEF was achieved using a helper-independent retroviral vector, RCAS, derived from Rous sarcoma virus (Hughes et al., 1987). In vitro mutagenesis was used to introduce a deletion of residues 6–41 in the cytoplasmic domain of the wild-type receptor (Rothenberger et al., 1987). The same mutation was also made in an existing mutant TR in which cysteines 89 and 98 had been converted to serines to eliminate the two intermolecular disulfide bonds between receptor subunits (Jing

and Trowbridge, 1987). A mutant receptor with a cytoplasmic domain of only four amino acids, amino-terminal Met-Met-Lys-Arg, was prepared by deletion of amino acids 3–59.

1–2 wk after transfection, virtually all cells displayed human TRs on their cell surface as a result of infection by recombinant virus. The level of expression of wild-type and mutant human TRs on infected CEF varied from 3.6–7.7 × 10^5 molecules/cell, with dissociation constants for the binding of human Tf ranging from 3.6–8.7 nM (Table I). About 10^5 chicken TRs were expressed on infected and uninfected CEF with a dissociation constant for the binding of ovotransferrin of ~100 nM, which is similar to previously reported values (Williams and Woodworth, 1973; Schmidt et al., 1986; Sorokin et al., 1987). Uninfected CEF did not bind human Tf.

Growth of CEF Expressing Wild-type and Mutant Human TRs

Growth of many cultured cells is dependent on Tf-mediated iron uptake (Barnes and Sato, 1980), but most mammalian Tfs do not support the growth of chicken cells (Shimo-Oka et al., 1986). We were able to test, therefore, whether mutant human receptors could mediate iron uptake by determining their ability to support growth of CEF under conditions in which mammalian Tf was the only source of iron. As shown in Fig. 1 a, CEF expressing the wild-type human TR grew well in tissue culture medium supplemented with 3% (vol/vol) horse serum and 50 μg/ml human Tf, whereas uninfected CEF did not grow. CEF expressing mutant human TRs with a 36–amino acid deletion in the cytoplasmic domain also grew, but more slowly than cells expressing the wild-type receptor; cells expressing human TR lacking all but four amino acids of the cytoplasmic domain grew poorly.

After ~6 d of growth, we detected rapidly growing foci of cells in cultures of CEF expressing mutant TRs. These cells were isolated and were now found to grow in the same medium as well as CEF expressing wild-type human TR (Fig. 1 b). Binding studies showed that cells selected under these growth conditions expressed more mutant human TRs than the original cell population. The level of expression of mutant human TRs with a partial deletion in their cytoplas-

Table I. Surface TR Expression on CEF Infected with Human TR Retroviral Constructs

Cells	Human TR cDNA retroviral construct	Human Tf surface binding sites ×10^-5	K_d
			nM
Wt	Wt	4.54 ± 0.36	3.95 ± 0.03
S8998	Cys[89.98]→Ser[89.98]	7.35 ± 1.16	8.69 ± 0.47
Wt 6-41a	Δ 6-41	7.73 ± 0.67	5.22 ± 0.37
Wt 6-41b	Δ 6-41	7.23 ± 1.42	6.67 ± 0.25
S6-41a	Δ 6-41, Cys[89.98]→Ser[89.98]	3.60 ± 0.28	3.56 ± 0.21
S6-41b	Δ 6-41 Cys[89.98]→Ser[89.98]	4.00 ± 0.56	4.87 ± 0.26
Wt 3-59	Δ3-59	4.19 ± 1.26	3.82 ± 1.11
CEF	None	<0.01	—

The number of Tf binding sites and K_d values on CEF infected with wild-type and mutant human TR retroviral constructs were determined as described in Materials and Methods. The values shown represent the mean values ± SEM of three to four experiments with cells from at least two different transfections. Cells labeled a and b were transfected with independently derived retroviral constructs. *Wt*, wild type.
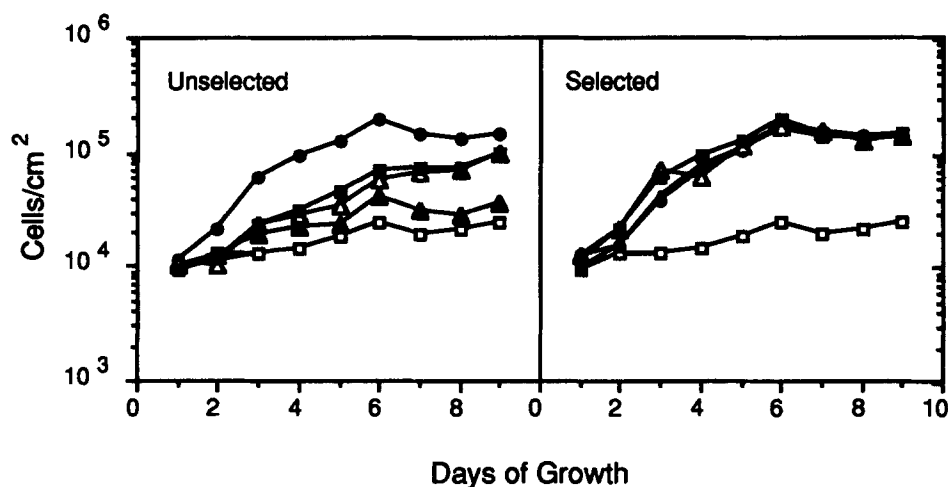
Figure 1. Growth of CEF expressing wild-type and mutant human TRs in tissue culture medium supplemented with human Tf. The growth curves shown in the left-hand panel are of unselected cells grown previously in DME supplemented with 1% defined calf serum, 1% chicken serum, and 2% tryptose phosphate broth. The growth curves shown in the right-hand panel are of cells previously selected by growth in DME supplemented with 3% horse serum, 50 μg/ml human Tf, and 2% tryptose phosphate broth. Other experimental details are given in Materials and Methods. Cells are CEF (□), wild type (●), Δ3-59 (▲), wild type 6-41a (■), and S6-41a (△).

mic domain was two- to threefold higher (from 5.8 to 13.3 × 10^5 and 6.0 to 15.9 × 10^5 receptors/cell for the C6-41a and S6-41a cells, respectively), whereas that of mutant human TRs with a cytoplasmic domain of only four amino acids was more than tenfold higher (from 1.1 to 12.6 × 10^5 receptors/cell). In contrast, the expression of wild-type human TRs on CEF grown under identical conditions was virtually unchanged (from 5.8 to 6.8 × 10^5 receptors/cell). Taken together, the results of the growth studies indicate (*a*) that the ability of mutant TRs with deletions in their cytoplasmic domain to mediate iron uptake is impaired; and (*b*) that, provided mutant receptors are expressed at high levels, they can transport sufficient iron to allow normal cell growth.

Kinetics of Internalization and Recycling of Wild-type and Mutant TRs

The growth studies reported above indicate that tailless mutant human TRs are internalized at a detectable rate in CEF. To obtain further information on the endocytosis of mutant and wild-type human receptors in CEF, we used a pulse–chase protocol in which cells were incubated at 4°C for 60 min with ^{125}I-labeled Tf, washed, and then incubated at 37°C. After various times, the amount of ^{125}I-labeled Tf bound to the cell surface, inside the cells, and released into the medium was determined using an acid wash to distinguish between cell surface–bound and internalized Tf (Hopkins and Trowbridge, 1983). In cells expressing wild-type human TRs, the acid-resistant, cell-associated, ^{125}I-labeled Tf representing the pool of internalized ligand increased to a peak of >35% of the total by 10 min and then declined; by 30 min, >90% of the ^{125}I-labeled Tf had been released into the medium (Fig. 2). The pool of acid-resistant Tf in cells expressing mutant receptors was much smaller than in cells expressing wild-type receptors, indicating that the internalization of the mutant receptors was greatly impaired. Nevertheless, Tf was released into the medium from cells expressing mutant TRs as rapidly as from cells expressing wild-type receptors as a result of the direct dissociation of the ligand from receptors on the cell surface. The rapid dissociation of Tf at 37°C from human TRs on the surface of

CEF is consistent with previous studies of a human hepatoma cell line (Ciechanover et al., 1983) and precludes direct measurement of the internalization rates of the mutant receptors.

The relative efficiency of internalization of wild-type and mutant receptors can be estimated, however, from measurements of the steady-state distribution of TRs and their rate of recycling at 37°C (Tanner and Lienhard, 1987). At steady state, the rate of internalization of the diferric Tf-TR complexes, $[TR]_{sur}$, equals the rate of recycling and externalization of the internal pool of apoTf-TR complexes, $[TR]_{int}$: $k_{int}[TR]_{sur} = k_{ext}[TR]_{int}$, assuming an insignificant rate of intracellular degradation of receptors. Thus, Fig. 4 is a minimum estimate of k_{int}. The values for $[TR]_{sur}$ and $[TR]_{int}$ can be obtained from steady-state binding of Tf under saturating conditions at 37°C. To determine these parameters experimentally, cells were incubated with ^{125}I-labeled human Tf for 15, 30, or 60 min at 37°C and then washed, and the fraction of the bound ligand resistant to removal by an acid wash was determined. As shown in Fig. 3, in cells expressing either mutant or wild-type receptors, steady-state conditions were reached after incubation with Tf for 30–60 min. Similar results were obtained for cells selected for high expression of mutant TRs as for unselected cells expressing two- to tenfold fewer receptors (Fig. 3). Of the Tf bound to cells expressing wild-type receptors, 62.2 ± 1.3% (*n* = 4; ±SEM) was inside the cell, whereas the fraction of Tf internalized in those expressing mutant receptors ranged from 15.4 to 19.2% for the unselected lines and 11.9 to 15.8% for the selected lines.

The rate at which internalized receptors recycle back to the cell surface was determined after selectively removing Tf bound to receptors on the cell surface under conditions that do not impair recycling of endocytosed Tf-TR complexes. This was achieved by loading cells at 37°C with ^{125}I-labeled Tf for 1 h to attain steady-state conditions and then removing iron from surface-bound Tf at 4°C using a deferoxamine wash as described in Materials and Methods. At pH 7.2 and 4°C, apoTf rapidly dissociates from TRs on the cell surface (Dautry-Varsat et al., 1983). As shown in Fig. 4, internalized ^{125}I-labeled Tf was released into the medium from cells expressing Δ3-59 mutant human TRs at a rate that was indistin-
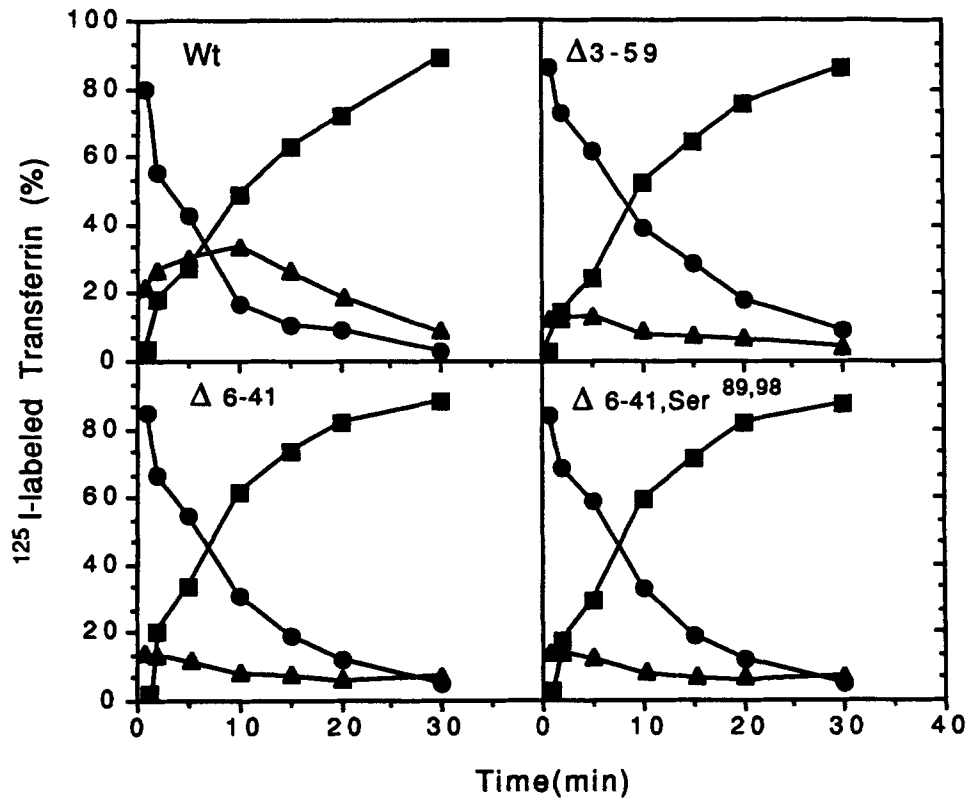
Figure 2. Kinetics of human Tf recycling in CEF expressing mutant and wild-type receptors. CEF expressing the human TRs indicated were preincubated at 4°C with ^{125}I-Tf, washed, and then incubated at 37°C for the times shown. An acid wash at pH 2.4 was used to distinguish surface-bound and internalized ^{125}I-Tf, as described in Materials and Methods. Surface-bound ^{125}I-Tf (●), internalized ^{125}I-Tf (▲), and ^{125}I-Tf released into the medium (■) were expressed as a percentage of the total radioactivity recovered. The points represent an average of triplicate determinations.

guishable from that of cells expressing wild-type receptors. After the first 2 min of incubation at 37°C, the release of Tf into the medium followed first-order kinetics, and the average values of the first-order rate constants for the recycling of the wild-type and mutant receptors were 0.056 ± 0.010 and 0.048 ± 0.006 min^{-1} (n = 4), respectively. We conclude, therefore, that deletion of residues 3–59 in the cytoplasmic domain of the human TR does not significantly affect the rate at which internalized receptors recycle back to the cell surface.

From the equation above, it can then be calculated that the relative efficiency of the mutant receptors (k_{int} mutant
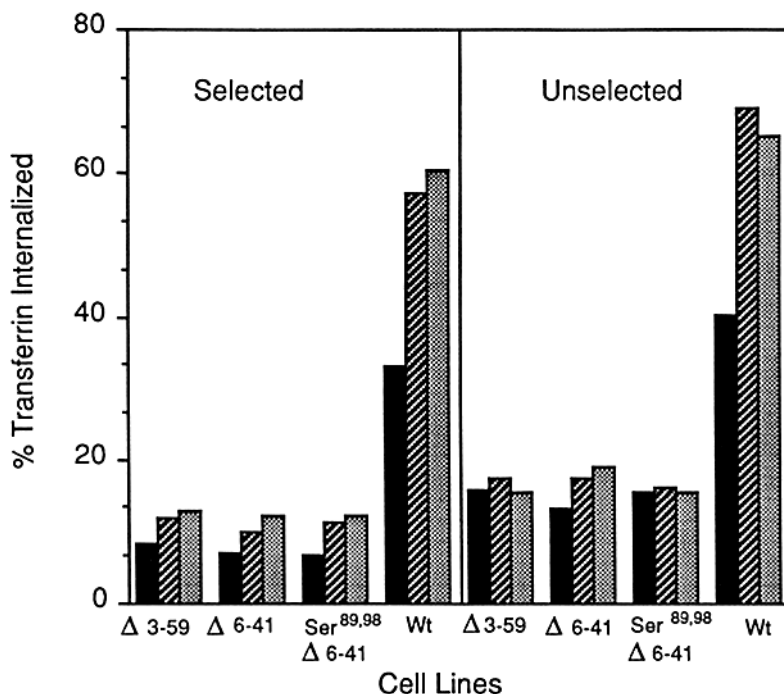

Figure 3. Distribution of cell surface and intracellular wild-type and mutant human TRs under steady-state conditions. CEF expressing the human TRs indicated were incubated with ^{125}I-labeled Tf for 15 (*solid bars*), 30 (*hatched bars*), and 60 min (*speckled bars*) at 37°C and washed, and the acid wash technique described in Materials and Methods was used to distinguish surface-bound and internalized Tf. The left-hand panel displays the results from cells selected for growth in mammalian Tf that express higher levels of mutant TRs (see text). The right-hand panel displays the results from cells grown under normal culture conditions in the presence of chicken serum. CEF bound <2% of the human Tf bound to any of the cells expressing human TRs.
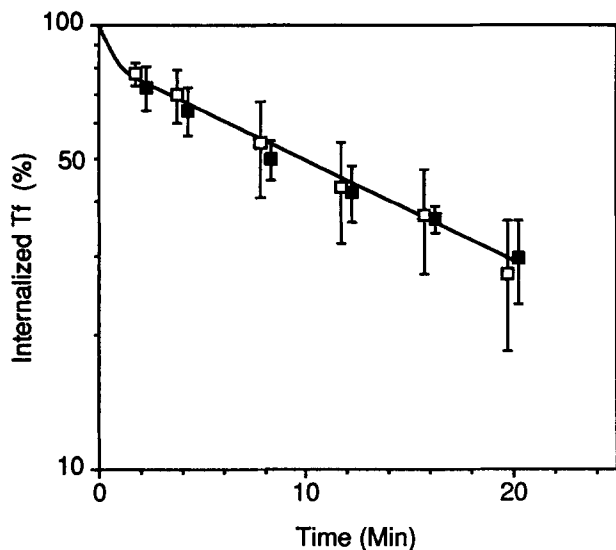
Figure 4. Rate of recycling of wild-type and Δ3-59 mutant human receptors. CEF expressing wild-type or Δ3-59 mutant human TRs were incubated with ^{125}I-labeled Tf for 1 h at 37°C, and surface-bound ^{125}I-labeled Tf was then removed by the deferoxamine wash procedure performed at 4°C as described in Materials and Methods. Cells were then incubated at 37°C for various times, and the radioactivity remaining associated with the cells and released into the medium were determined. The results shown represent the mean values ± SEM from four independent experiments of the percentage of internalized ^{125}I-labeled Tf remaining in CEF expressing wild-type (□) or Δ3-59 mutant (■) human TRs.

TR/k_{int} wild-type TR × 100%) expressed on the unselected and selected CEF range from 11.0 to 14.3 and 9.0 to 12.9%, respectively.

Labeling with Gold-Antibody Complexes and Tf-Peroxidase

The human TR-specific monoclonal antibody, B3/25, coupled to colloidal gold, was used to investigate the distribution of wild-type and mutant TRs on the surface of CEF and their uptake. Cells were incubated with human Tf at 10°C for 30 min, washed, fixed, and then incubated with gold-labeled anti-TR antibody and processed for whole mount electron microscopy as described previously (Hopkins and Trowbridge, 1983). In the case of both the wild-type and Δ3-59 tailless mutant human TRs, clusters of receptors could be found associated with coated pits on the cell surface (Fig. 5, *a*, *b*, and *inset*, and Fig. 6).

To follow the uptake of Tf, cells were incubated with human Tf-peroxidase. When cells expressing the mutant human receptors with a deletion of amino acids 3–59 were incubated with human Tf-peroxidase for 30 min at 5°C, rinsed, then warmed at 37°C for 10 min before fixation, reaction product was found in coated pits and in intracellular vesicles typical of the endocytic pathway (Fig. 6).

In addition to coated pits, a variety of endocytic invaginations, including 50-nm omega-shaped uncoated pits and sites of viral binding, were also observed in the course of these morphological studies. Gold–antibody complexes and Tf-peroxidase reaction products were only rarely associated with these structures.

The Role of Chicken TRs in the Endocytosis of Human Tf

Although chicken TRs do not bind mammalian Tf and, therefore, cannot directly internalize human Tf, the question arises whether chicken receptors might form complexes with human TRs that account for the ability of mutant human TRs to be internalized. Analysis of deletion mutants in which cysteines 89 and 98 were converted to serines, thus eliminating the formation of intermolecular disulphide bonds between subunits (Table I; Jing and Trowbridge, 1987), demonstrated that covalent association of chicken and human TRs is not required for endocytosis of mutant human receptors. However, this does not rule out the possibility that chicken and human TRs are noncovalently associated. Immunoprecipitation studies indicate that, if such complexes exist, they do not survive solubilization in the detergent solutions used to lyse cells since no chicken TRs coprecipitate with mutant human receptors under conditions in which small numbers of chicken receptors would be detected (data not shown).

Electron microscopy of prefixed cells in which both mutant human TRs and chicken TRs were selectively labeled also argues against the distribution of human TRs being influenced by chicken TRs. Thus, when CEF expressing mutant human TRs with a deletion of amino acids 3–59 in their cytoplasmic domain were incubated with human Tf for 30 min at 10°C, fixed, and labeled with mAbs against the human and chicken TRs coupled to gold particles that differed in size, pits containing only closely packed human TRs were common (Fig. 7). As cells were fixed before staining with antibody–gold complexes, the clustering of human receptors into coated pits was not induced by cross-linking with the multimeric gold-antibody complexes.

Localization of a Signal Sequence for Internalization within the Cytoplasmic Domain of the Human TR

The cytoplasmic domain of the human TR clearly plays an important role in facilitating rapid endocytosis. To determine whether this function could be localized to a specific region of the cytoplasmic domain, a series of TR mutants were generated with different deletions in the cytoplasmic domain. As shown earlier, deletion of almost the entire cytoplasmic domain of the receptor does not significantly affect the rate of receptor recycling, so that differences in the steady-state intracellular levels of mutant receptors would be expected to reflect primarily differences in their internalization rates. Mutant receptors were found to fall into two classes; receptors with deletions of amino acids 3–29, 3–35, and 6–41 had similar steady-state distributions to the Δ3-59 tailless mutant receptor, whereas the steady-state distributions of receptors with deletions spanning amino acid residues 3–18, 29–59, and 42–59 were comparable with that of the wild-type receptor (Fig. 8). These results suggested that one or more of the ten amino acids at positions 19–28 are required for high efficiency internalization of the human TR.

To confirm this conclusion and to investigate whether amino acid residues 19–28 are sufficient for rapid internalization, a second deletion encompassing amino acids 29–59 was introduced into the deletion mutant already lacking amino acid residues 3–18. In effect, this inserts amino acids 19–28 into the truncated cytoplasmic domain of the Δ3-59 tailless receptor. We then determined the steady-state distri-
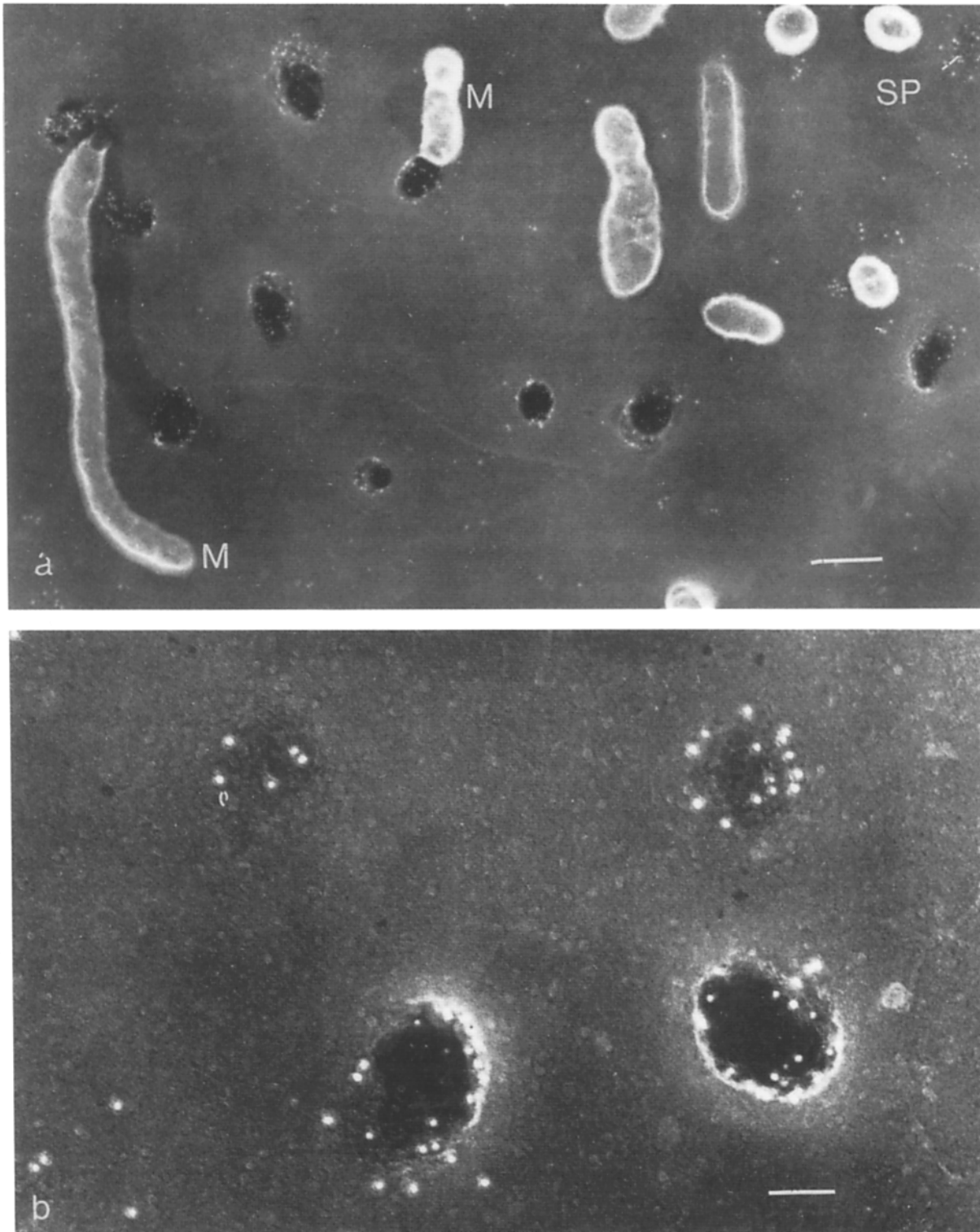
Figure 5. Cells expressing wild-type human TRs labeled with 8-nm gold complexes. (*a*) Low power view showing the form and distribution of pits. All of the pits contain gold complexes. Gold complexes (sometimes in clusters) are also distributed on the cell surface. *SP,* shallow pit; *M,* microvilli. Whole mount. (*b*) Higher power showing that, while some pits are deeply invaginated, others are shallow and are presumably at an early stage of formation. Rotary shadowing puts a halo around particles exposed on the cell surface and in the shallow pits. Whole mount. Bars: (*a*) 300 nm; (*b*) 50 nm.
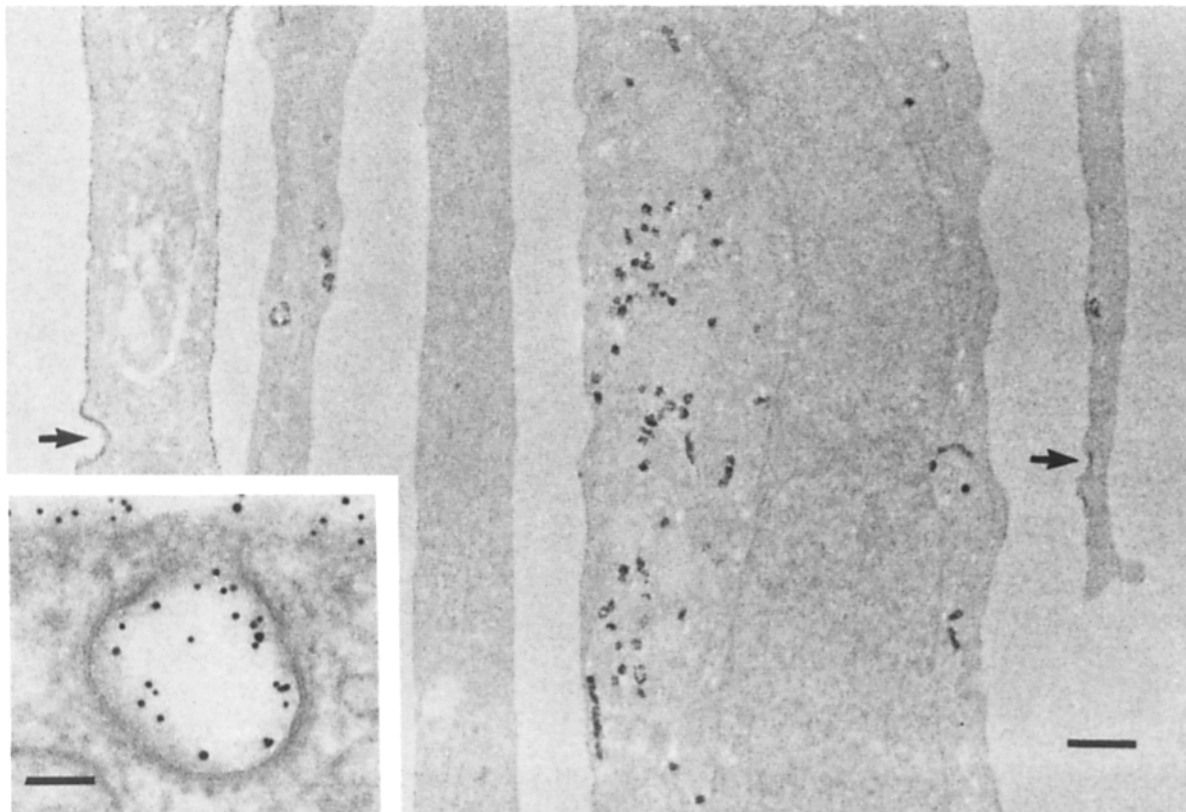
Figure 6. Uptake of human Tf-peroxidase by CEF expressing (Δ3-59) mutant human TRs. A section through a cell expressing mutant human TRs with a deletion of amino acids 3-59 in the cytoplasmic domain showing the ability of these receptors to internalize human Tf-peroxidase after binding at 5°C. The cells which have been warmed to 37°C for 30 min show label on the surface (some within pits) and internally (within the endocytic pathway). Cells had been selected for high expression of mutant human TRs by growth in mammalian Tf as described in the text. Conventional epon section. (*Inset*) Cells expressing (Δ3-59) mutant human TRs and labeled with 8-nm gold complexes. The endocytic invaginations into which the TRs become concentrated (shown in Fig. 1) are typical coated pits. Bars: 200 nm; (*inset*) 50 nm.

bution and the value for the rate constant for externalization (k_{ext}) of these mutant receptors. The percent of receptors internalized at steady-state was 79.0 ± 1.1 ($n = 5$), and the value for k_{ext} was 0.029 ± 0.013 ($n = 4$). From these data, the internalization efficiency of the mutant TRs was estimated to be 88.2%, consistent with the notion that the region of the cytoplasmic domain of the human TR spanning residues 19-28 contains a signal sequence that is both required and sufficient for high efficiency internalization.

In view of the evidence that tyrosine residues within the cytoplasmic domain are especially critical for rapid internalization of other proteins (Davis et al., 1987; Pearse, 1988; Lazarovits and Roth, 1988; Lobel et al., 1989), it was of interest that the only tyrosine residue in the cytoplasmic domain of the human TR is located at position 20 and, therefore, is included within the region indentified as containing a signal sequence for rapid endocytosis. To investigate whether Tyr 20 of the human TR is important for rapid endocytosis, a mutant receptor was constructed in which this tyrosine residue was changed to glycine. We found that 30.0 ± 2.9% ($n = 4$) of the mutant receptors containing glycine at position 20 were intracellular under steady-state conditions and that the value of K_{ext} for this mutant TR was 0.047 ± 0.008 ($n = 4$). From these data, the internalization efficiency of the Tyr[20]→Gly[20] mutant TR was calculated to be 19.7%.

[59]Fe Uptake Experiments

Measurement of cellular ^{59}Fe accumulation provides an independent method of measuring the steady-state rate of internalization and recycling of TRs. The rate of iron uptake is proportional to the number of functional surface TRs so that values of cellular ^{59}Fe uptake need to be normalized for differences in the surface expression of wild-type and mutant receptors in cultures of CEF to estimate their relative efficiency of internalization. A representative experiment in which the rate of cellular ^{59}Fe uptake from human Tf by CEF expressing either wild-type or various mutant human TRs was determined is shown in Fig. 9. Cells expressing mutant human TRs containing glycine at position 20 accumulated ^{59}Fe from human Tf at almost the same rate as cells expressing wild-type human TRs but expressed almost fivefold more receptors. CEF expressing other mutant human receptors accumulated iron more slowly but at rates higher than control CEF. The relative rates of ^{59}Fe uptake by wild-type and mutant TRs normalized for differences in levels of receptor expression on the various cell lines are given in Table II.

Discussion

We have described an experimental system to study the functional activity of the human TR in which a helper-indepen-
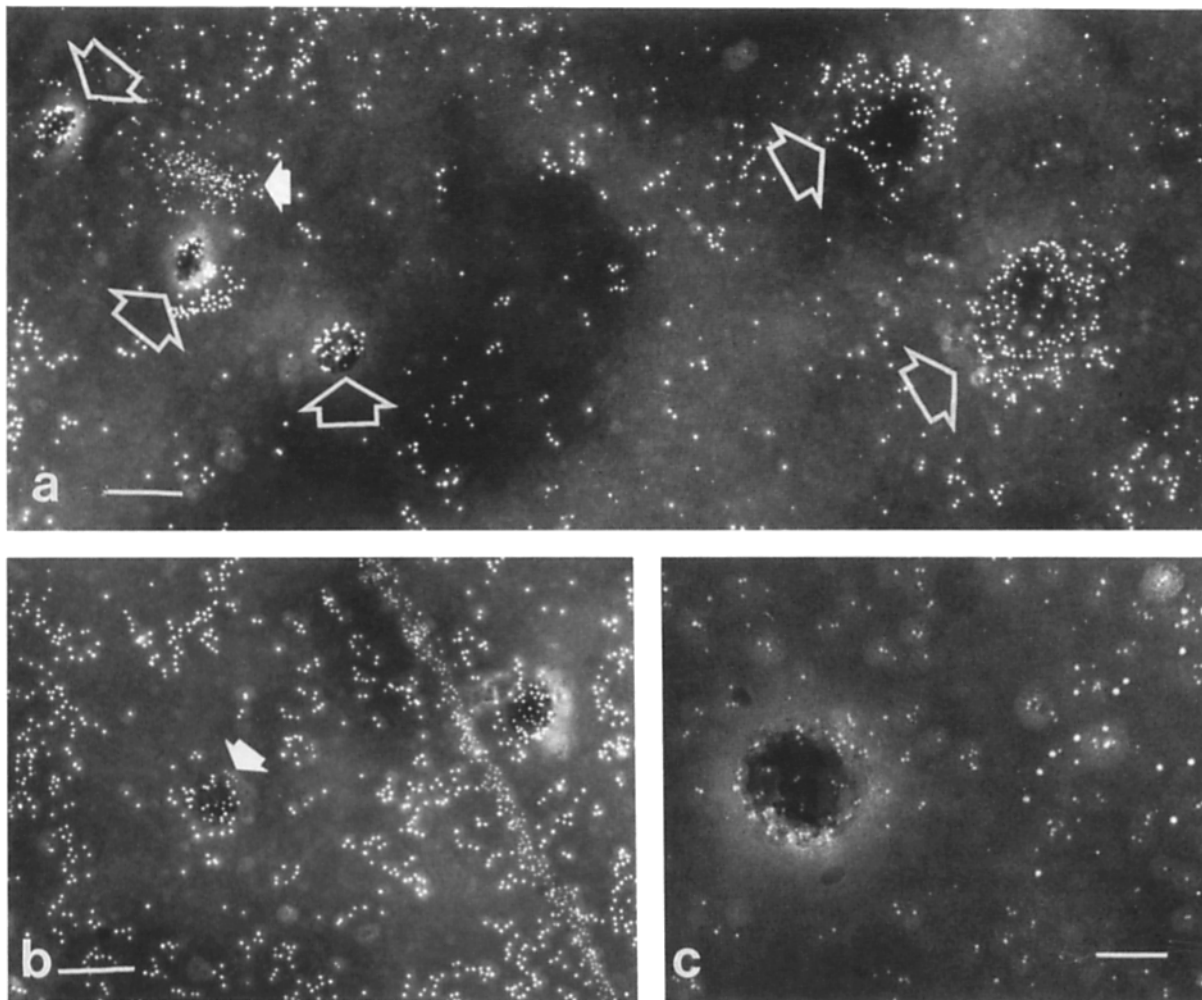
Figure 7. Double labeling of human and chicken receptors on CEF expressing wild-type chicken TRs and mutant human TRs. (*a*) Human TRs labeled with 8-nm gold complexes and chicken TRs labeled with 5-nm gold complexes. Shallow and deep pits are indicated by large arrows. The gold complexes associated with the pits are all 8 nm diameter (i.e., human TR). Occasionally, the particles look slightly smaller, but this is because they lie deep within the pit cavity. Away from the pit, mixed aggregates of large (human TR) and small (chick TR) complexes can be seen (*small solid arrow*). In general, the relative number of human and chicken TRs identified by different sized gold complexes reflects the numbers assayed by radiolabeled ligand binding. However, it should be noted that the efficiency of labeling with gold complexes is significantly more sensitive with smaller (5 vs. 8 nm) particles. (*b*) Similar to *a*. Here, pits containing both human and chicken TR are present (small arrows indicate chicken TR on the rim of a pit), their smaller diameter (5 nm) clearly evident. Note linear array of chicken TR that is probably associated with extracellular matrix; transfected human TRs never show this distribution. (*c*) Complete deletion (Δ3-59) mutant of human TR. Human TR are labeled with 5-nm gold complexes, while chicken TR are labeled with 8-nm complexes. The pit contains only human transferrin receptors. Bars: (*a*) 100 nm; (*b*) 100 nm; (*c*) 80 nm.

dent retroviral vector directs high level expression of the receptor in CEF. The system has two major advantages: first, as infectious virus is produced by cells transfected with the retroviral vector containing the human TR cDNA, cultures of CEF in which virtually all cells express recombinant human TRs can be obtained within 1–2 wk after transfection without the need for growth in selective drugs; and, second, chicken TRs do not bind human Tf, so that binding and internalization of human Tf by recombinant human receptors expressed in CEF can be determined without a direct contribution to the assay by resident wild-type chicken receptors. In addition, the functional status of mutant human TRs can be assessed by growth assays in medium supplemented with mammalian Tf, and, by prolonged growth under these conditions, cells expressing high levels of receptors can be selected.

In agreement with a previous study by Rothenberger et al.

(1987), we find that the cytoplasmic domain of the human receptor is required for high efficiency endocytosis. These investigators concluded that the internalization of the human TR in mouse L cells was abolished by deletion of amino acids 6–41 in the cytoplasmic domain (Rothenberger et al., 1987; Iacopetta et al., 1988). However, our results indicate that, in CEF, internalization of mutant human TRs with the identical deletion of amino acids 6–41 and mutant receptors lacking virtually the entire cytoplasmic domain was substantially impaired but not abrogated. The most likely explanation for this disparity between the two studies is that, despite their relative abundance, a low level of Tf internalization by mutant human TRs in the mouse L cell transfectants examined by Rothenberger et al. (1987) could have been obscured by the background internalization of human Tf by endogenous mouse TRs.

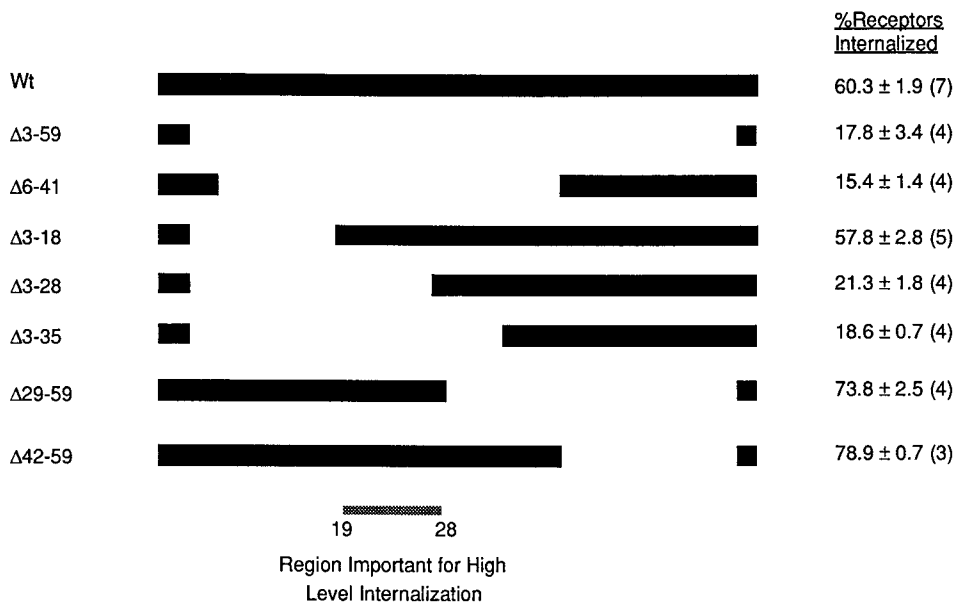The ability of mutant human receptors essentially lacking

%Receptors
Internalized

Wt   60.3 ± 1.9 (7)

Δ3-59   17.8 ± 3.4 (4)

Δ6-41   15.4 ± 1.4 (4)

Δ3-18   57.8 ± 2.8 (5)

Δ3-28   21.3 ± 1.8 (4)

Δ3-35   18.6 ± 0.7 (4)

Δ29-59   73.8 ± 2.5 (4)

Δ42-59   78.9 ± 0.7 (3)

19   28
Region Important for High
Level Internalization

Figure 8. Deletional analysis of the region of the human TR required for high efficiency endocytosis. The percentage of human TRs internalized for each of the deletion mutants shown was determined exactly as described in the legend to Fig. 3. The values shown represent the average ± SEM of the number of determinations shown in parentheses.

all of the cytoplasmic domain to supply sufficient iron from human Tf to support the growth of CEF establishes unequivocally that the receptors are being internalized and recycled at a finite but low rate. We have estimated the efficiency of internalization of the mutant receptors by two independent
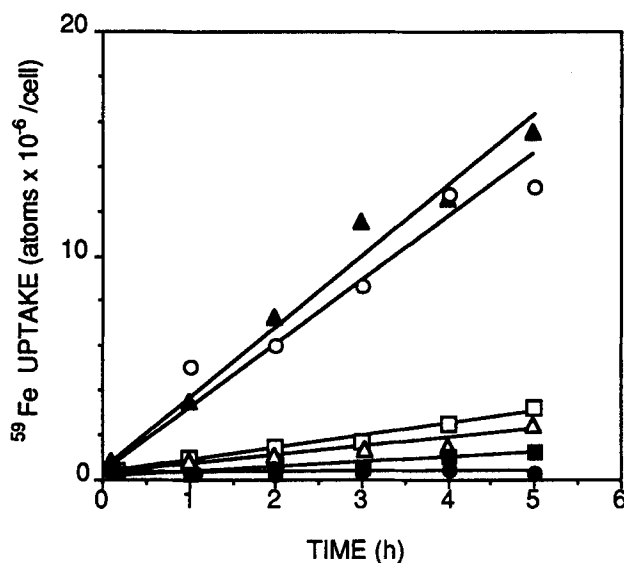

TIME (h)

Figure 9. Uptake of ^{59}Fe from human Tf by CEF expressing wild-type or mutant human receptors. Uptake of ^{59}Fe from human Tf by CEF expressing human TRs was determined by incubating cells with ^{59}Fe-Tf for the times indicated and then washing the cells and determining their radioactivity. The results shown are for a representative experiment, and each point represents the average values of ^{59}Fe uptake by triplicate cultures of CEF (●) and CEF expressing wild type (▲), Tyr^{20}→Gly^{20} (○), Δ3-18 and Δ29-59 (□), Δ3-59 (■), and Δ6-41 (△) mutant human receptors. The results are expressed on a per cell basis and have not been normalized for differences in receptor levels. The relative levels of human TRs on the surface of the various CEF used in this experiment, with wild-type receptors set at 1.0, were 4.9, 0.22, 0.7, and 1.6 for the Tyr^{20}→Gly^{20}, Δ3-18, Δ29-59, Δ3-59, and Δ6-41 mutant receptors, respectively.

methods: (*a*) measurement of the steady-state distribution of receptors and their recycling rate; and (*b*) determination of the rate of iron accumulation from human Tf by CEF expressing recombinant human TRs. After normalization for different receptor levels on the various cell lines, the latter method gives an estimate of relative rates of internalization and recycling equivalent to the internalization index previously used to evaluate the internalization efficiency of receptors that do not recycle their ligands back to the cell surface (Goldstein et al., 1977; Lobel et al., 1989). Both methods gave similar results, indicating that mutant human TRs with deletions of either amino acids 6-41 or 3-59 in their cytoplasmic domain are both internalized ∼10% as efficiently as wild-type receptors. The marginally higher values of internalization efficiency obtained from measurements of the steady-state distribution of human TRs at 37°C may reflect the possibility that there is a low background of surface Tf not removed by the acid wash technique that might lead to an overestimate of internalized mutant TRs.

The basis for the residual low rate of internalization of mutant human TRs lacking virtually all the cytoplasmic domain has not been established conclusively in the present studies. Expression of mutant human TRs in CEF precludes a direct

Table II. Efficiency of ^{59}Fe Uptake by Wild-type and Mutant Human TRs Expressed in CEF

Human TR constructs	Relative ^{59}Fe uptake*
	%
Wt	100
Δ6-41	7.2 ± 0.3
Δ3-59	6.0 ± 0.7
Δ3-18, Δ29-59	94.7 ± 12.6
Tyr^{20}→Gly^{20}	17.5 ± 0.7

* ^{59}Fe uptake from human ^{59}Fe-Tf by CEF expressing wild-type or mutant human TRs was determined as described in Materials and Methods. The rates of iron uptake were normalized for differences in the levels of human receptors on the surface of the various sets of cells and then expressed as a percentage relative to uptake by wild-type human receptors. The results represent the mean values ± SEM from three independent experiments. *Wt*, wild type.

LDL Receptor NH₂ ~~~~

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        + + +      - ■ +    ---       -        +       ---
KNWRLKNINS INFDNPVYQK TTEDEVHICH NQDGYSYPSR QMVSLEDDVA
++ + +    +   - ----    + -     - - +    + ■  -        + -
RKPKTVNAKT NNDANEEEDV ALKMEVHSND GDVQRALSFR TYSLPEGGFL NSFASRAQDM M
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TR COOH ~~~~

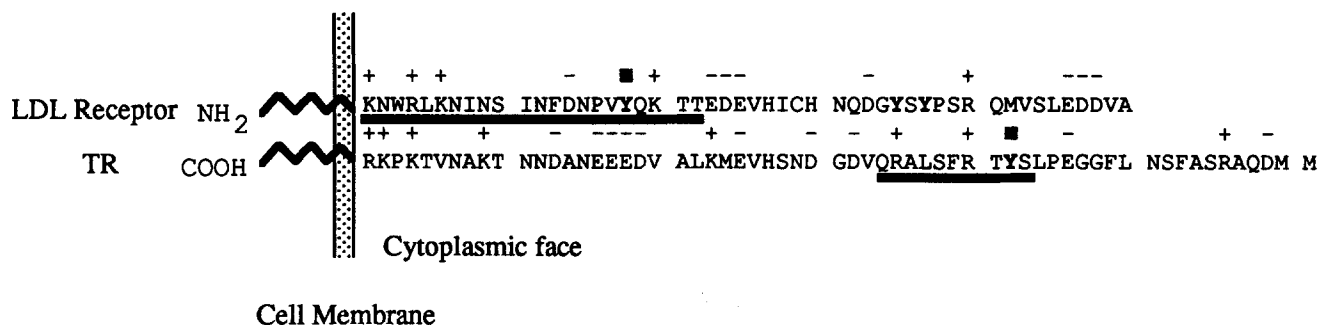Cytoplasmic face

Cell Membrane

Figure 10. A comparison of the amino acid sequences of the human TR and LDL receptor required for high efficiency endocytosis. The regions of both receptors required for high efficiency endocytosis are underlined. Basic and acidic amino acids are indicated by the positive and negative signs, respectively. The tyrosine residue in the cytoplasmic domain of the LDL receptor important for internalization and the only tyrosine residue in the cytoplasmic domain of the TR are indicated by the solid square. The LDL receptor and TR sequences are oriented with respect to the cell membrane so that the termini shown represent the carboxy- and amino-terminal sequences, respectively. Data for the LDL receptor is from Davis et al. (1987).

role for endogenous wild-type receptors since they do not bind human Tf. However, this does not exclude that chicken TRs could indirectly facilitate the internalization of human Tf by forming complexes with the mutant human TRs. The strongest evidence against this possibility is that double labeling with gold complexes showed that tailless mutant human receptors can be found clustered within coated pits in the absence of chicken TRs. The fact that the steady-state distribution of mutant human TRs is largely independent of the number of receptors expressed over a more than tenfold range also argues against a role for chick TRs in their internalization (Fig. 3). From the results of the morphological studies presented in Figs. 5–7, it seems clear that the mutant receptors are not excluded from coated pits and can be taken up into endosomes. However, whether the tailless mutant human TRs are concentrated in coated pits rather than randomly included and whether endocytosis via coated pits is the major pathway by which they are internalized requires further study. The results shown in Fig. 4 indicate that at least a large fraction of the internalized tailless mutant human TRs recycle back to the cell surface at a rate indistinguishable from wild-type receptors. This implies that the cytoplasmic domain does not greatly influence the rate of reappearance of TRs at the cell surface.

The experimental system we have described has enabled us to localize a structural determinant for high efficiency internalization to a 10–amino acid region encompassing residues 19–28 of the human TR cytoplasmic domain. It has previously been shown that the first 22 amino acids of the LDL receptor cytoplasmic domain contiguous with the transmembrane region are sufficient for high efficiency endocytosis (Davis et al., 1987). Thus, it appears likely that the common structural feature of the cytoplasmic domains of these two receptors that determines efficient internalization is contained within a short stretch of contiguous amino acids analogous to the targeting sequences that determine the trafficking pathways of newly synthesized proteins and their segregation into appropriate cellular organelles (for reviews see Pfeffer and Rothman, 1987; Robinson and Austen, 1987; Verner and Schatz, 1988).

A comparison of the amino acid sequences of the cytoplasmic domains of the TR and LDL receptor which are in opposite orientation with respect to the plasma membrane are shown in Fig. 10. It is of interest that the region of the cytoplasmic domain of the human TR (residues 19–28) required for high efficiency endocytosis is separated from the transmembrane region of the receptor by 33 amino acids. Thus, if this region is physically located near the cytoplasmic face of the plasma membrane, as must be the functionally equivalent region of the LDL receptor, then the cytoplasmic domain of the TR would have to adopt a tertiary structure that juxtaposes residues 19–28 in proximity to the cell membrane.

Recent studies of the LDL receptor, mannose-6-phosphate receptor, and a mutant influenza virus hemagglutinin have shown that tyrosine residues within the cytoplasmic domains of these proteins are especially critical components of the recognition feature that determines high efficiency endocytosis (Davis et al., 1987; Lazarovits and Roth, 1987; Lobel et al., 1989). The single tyrosine residue in the cytoplasmic domain of the human transferrin receptor is located at position 20 within the 10–amino acid region of the cytoplasmic domain we identified as important for rapid internalization. Analysis of a mutant human TR containing glycine at position 20 indicates that tyrosine is also an important element of the determinant within this region that determines high efficiency internalization. The internalization efficiency of this mutant receptor, however, is significantly higher than the Δ6-41 and Δ3-59 deletion mutants, consistent with the notion that the tyrosine residue is part of a more complex signal sequence for internalization. It should be noted that deletions of residues 29–59 and 42–59 in the cytoplasmic domain of the human TR that change the position of Tyr 20 relative to the transmembrane region of the receptor did not markedly influence internalization efficiency.

Clathrin-coated pits at the plasma membrane contain a protein complex composed of 100-, 50-, and 16-kD components termed accessory proteins or adaptors (Pearse and Crowther, 1987; Robinson, 1987; Keen, 1987; Manfredi and Bazari, 1987; Virshup and Bennett, 1988; Ahle et al., 1988). Recent work by Pearse and her colleagues suggests that the cytoplasmic domains of receptors that are rapidly internalized interact specifically with the adaptor protein complex (Pearse, 1988; Glickman et al., 1989). Although there is evidence that HA-II adaptor complexes that are localized to coated pits of the plasma membrane recognize the cytoplasmic domains of the LDL, poly Ig, and mannose-6-phosphate

receptors, a direct interaction between adaptor protein complexes and the TR has not yet been demonstrated. The components of adaptor complexes that are the most likely candidates to display a recognition site for a receptor signal sequence are members of the polymorphic family of 100-kD adaptins (Pearse, 1988), some of which are differentially localized to either plasma membrane or Golgi-associated coated membranes (Robinson, 1987; Ahle et al., 1988). Several distinct adaptins have recently been cloned (Robinson, 1989; Kirchhausen et al., 1989), and this should facilitate future studies of whether any of these proteins bind to the region of the human TR cytoplasmic domain that determines high efficiency endocytosis.

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