

# Mutagenesis of the Human Transferrin Receptor: Two Cytoplasmic Phenylalanines Are Required for Efficient Internalization and a Second-site Mutation Is Capable of Reverting an Internalization-defective Phenotype

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**Abstract.** Site-specific mutagenesis has been used to define the sequences required for efficient internalization of the human transferrin receptor. It has previously been shown that the sole cytoplasmic tyrosine, at position 20, is required for efficient internalization. When two other cytoplasmic aromatic residues, the phenylalanines at positions 13 and 23, are substituted with alanines internalization is also reduced. The phenylalanine 23 mutation decreases the internalization rate constant approximately threefold, and mutation of phenylalanine 13 decreases it by approximately twofold. The mutation at position 23 has as serious an effect on internalization as substitution with a nonaromatic amino acid for the single tyrosine. These results demonstrate the importance of several aromatic amino

acids in maintaining efficient internalization of the transferrin receptor. Substitution of a tyrosine at a second site, for a serine at position 34, within the cytoplasmic domain of a transferrin receptor with a nonaromatic amino acid at position 20, results in a complete reversion of the internalization-defective phenotype. This reversion is completely dependent upon a tyrosine, as phenylalanine substituted at position 34 does not revert the internalization-defective phenotype. This result demonstrates that a tyrosine placed outside of its native context can still function in the internalization of the transferrin receptor, suggesting a flexibility in surrounding sequences required for efficient internalization.

RECEPTOR-mediated endocytosis is a process used for the efficient internalization of a number of macromolecules. Included in the list of ligands taken up by endocytosis are ligands which fulfill nutritional requirements (e.g., transferrin-mediated iron accumulation) as well as ligands responsible for transmitting signals (e.g., epidermal growth factor). The hallmark of receptor-mediated endocytosis is the internalization of these disparate receptor-ligand complexes through clathrin-coated pits. Only a specific subset of plasma membrane proteins are efficiently clustered in and thereby internalized through coated pits, suggesting that these proteins contain specific clustering signals. Since a variety of receptors are known to be internalized through a single-coated pit (Dickson et al., 1981; Willingham et al., 1981; Carpentier et al., 1982), it is likely that a common clustering signal exists. The sequences of a large number of receptors (>25) have been determined, but examination of the predicted amino acid sequences has not revealed a cytoplasmic sequence motif, found in all receptors, that could potentially be assigned as the clustering or internalization signal. This observation suggests that the clathrin-coated pit clustering signal is more complex than a simple linear stretch of amino acids. Furthermore, although it is compelling to consider one clathrin-coated pit internalization signal it is

also possible that there are a number of "signals" that can function in this process (Verrey et al., 1990).

The only known general feature for efficient internalization through coated pits is the presence of a tyrosine on the cytoplasmic domain of the receptor. The requirement for a tyrosine in maintaining efficient internalization was first shown for the human low density lipoprotein (LDL) receptor (Davis et al., 1986), and has since been extended to include the human transferrin receptor (McGraw and Maxfield, 1990; Alvarez et al., 1990; Jing et al., 1990), the polymeric immunoglobulin receptor (Breitfeld et al., 1990), and the 275-kD mannose-6-phosphate receptor (Lobel et al., 1989). The functional significance of the cytoplasmic tyrosine in clathrin-coated pit endocytosis has not been determined.

It has recently been shown that amino acids near the tyrosine influence internalization of the LDL receptor (Chen et al., 1990). The sequence near the required tyrosine of the LDL receptor is asparagine-proline-valine-tyrosine. Mutagenesis of the LDL receptor, by substitutions of native residues with alanines, has shown that the asparagine and proline are required for efficient internalization whereas the

1. *Abbreviations used in this paper:* LDL, low density lipoprotein; S/I, surface/internal; TR, transferrin receptor.

valine is not. This amino acid sequence, NPXY, is found in the cytoplasmic domains of 13-membrane proteins and thus potentially could be a clathrin-coated pit clustering signal. However, a number of receptors that are internalized through coated pits do not contain this sequence (Chen et al., 1990). Therefore, although the presence of a cytoplasmic tyrosine appears to be a common requirement for efficient internalization, the NPXY motif cannot be the only clustering signal.

The role of the single cytoplasmic tyrosine of the human transferrin receptor (TR) for efficient internalization has been studied. Efficient internalization requires an aromatic amino acid at this position, rather than there being a stringent requirement for tyrosine, because both phenylalanine and tryptophan can be substituted for tyrosine without a significant effect on internalization (McGraw and Maxfield, 1990). The TR does not contain obvious homology with the cytoplasmic domains of other receptors and specifically does not contain the NPXY sequence.

To further define the requirements for efficient internalization of the human TR we have extended our *in vitro* mutagenesis studies of the cytoplasmic domain. In this paper we show that substitution of the phenylalanine at position 23 (see Fig. 1 *a*) with alanine results in a threefold reduction in the internalization constant. This effect is similar to that observed when the tyrosine at position 20 is substituted with a nonaromatic amino acid (McGraw and Maxfield, 1990). Substitution of the phenylalanine at position 13 with alanine results in a smaller effect on the internalization rate constant. These results demonstrate that aromatic amino acids, other than the tyrosine, are required for efficient internalization of the human TR.

To further explore the influence of the local amino acid environment on the functioning of the tyrosine in internalization, we have replaced the native tyrosine with a cysteine and substituted a tyrosine for the native serine at position 34. Surprisingly, this double mutant is internalized as efficiently as the wild-type receptor. This result demonstrates that a second-site mutation is capable of reverting the internalization defective phenotype of the cysteine 20 TR. Unlike the requirement for an aromatic amino acid at position 20 of the TR, reversion of the internalization-defective phenotype of the cysteine 20 receptor requires a tyrosine, as a cysteine 20-phenylalanine 34 double mutant is as internalization-defective as the single cysteine 20 mutation.

## Materials and Methods

### Cells

The CHO cell line, TRVb, that does not express detectable levels of functional hamster transferrin receptor was used as the recipient cell line in all transfections (McGraw et al., 1987). The behavior of the wild-type and *in vitro* mutagenized human TR expressed in TRVb cells have been previously reported (McGraw et al., 1987, 1988; McGraw and Maxfield, 1990). All tissue culture media and supplements were purchased from Gibco Laboratories (Grand Island, NY).

### Ligands

Human apotransferrin was purchased from Sigma Chemical Co. (St. Louis, MO) and further purified by Sephacryl S-300 gel filtration before use. Diferric transferrin, iodinated diferric transferrin and  $^{59}\text{Fe}$ -loaded transferrin were prepared as described previously (McGraw et al., 1987).

## *In Vitro* Mutagenesis

Site-directed mutagenesis was carried out according to the protocol of Kunkel (1985). The oligonucleotides used in this study were purchased from Operon Technologies, Inc. (San Pablo, CA). The nucleotide changes were confirmed by Sanger sequencing across the mutated site.

Complete human TR cDNA clones were reconstructed containing the *in vitro*-altered fragments as previously described (McGraw et al., 1988). The mutations were confirmed in the dsDNA used for transfection by sequence analysis. The altered receptors were introduced into TRVb cells by Lipofectin<sup>™</sup> (Bethesda Research Laboratories, Gaithersburg, MD). In all cases pSV3-Neo was cotransfected into the cells as a dominant selectable marker.

## Transferrin Receptor Endocytosis Assays

The assays used to characterize the surface/internal distribution, internalization, externalization, and Fe-accumulation rates of the TR have been described in detail elsewhere (McGraw and Maxfield, 1990).

The internalization rate constant was determined using a modification of the *In/Sur* plot (Wiley and Cunningham, 1982). Monolayers of cells grown in six-well plates ( $\sim 10^6$  cells/well) were washed and incubated in McCoy's growth salts supplemented to 26 mM  $\text{NaHCO}_3$  and 20 mM Hepes, pH 7.4 (med 1) for 15 min at 37°C in 5%  $\text{CO}_2$ . The cells were washed and incubated in med 1 supplemented to 2 mg/ml ovalbumin and 3  $\mu\text{g/ml}$   $^{125}\text{I}$ -transferrin ( $\sim 35$  nM) at 37°C in 5%  $\text{CO}_2$ . One six-well plate was processed per time point. After the desired incubation period (2, 4, 6, and 8 min) the cells were placed on ice, the incubation media removed and the monolayers flooded with 0.2 N acetic acid in 0.2 M NaCl (harsh acid wash) prechilled to 4°C. After a 2-min incubation on ice the cells were washed three times with med 2 (150 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM  $\text{CaCl}_2$ , 5 mM KCl, 1 mM  $\text{MgCl}_2$ ) prechilled to 4°C, solubilized in 0.1% Triton X-100, 0.1 N NaOH and counted in a gamma counter. The harsh acid wash removes surface bound ligands, thus cell-associated radioactivity after this wash has been internalized during the incubation at 37°C. The amount of surface TR was determined by incubating a six-well plate of cells with 3  $\mu\text{g/ml}$   $^{125}\text{I}$ -transferrin on ice, followed by five washes with med 2 prechilled to 4°C. The rate constant of internalization was determined as the slope of a plot of the ratio of internalized (acid resistant) transferrin/total surface transferrin binding versus time. The internalization rate constant is the fraction of surface TRs internalized per minute. For this modified *In/Sur* plot procedure to accurately measure the internalization rate constant the amount of transferrin-TR complex on the surface must remain constant over the course of the experiment (Wiley and Cunningham, 1982). We have previously shown this to be the case (McGraw and Maxfield, 1990), which is consistent with the TR being constitutively internalized (Hopkins and Trowbridge, 1983; Ajioka and Kaplan, 1986).

The rate at which internalized apo-transferrin is released from cells is used as a measure of the externalization rate constant. After an incubation for 1.5 h with 3  $\mu\text{g/ml}$   $^{125}\text{I}$ -transferrin, the cells were washed once with med 1 and incubated for 2 min in 50 mM MES, pH 5.0, 280 mM sucrose at room temperature. The monolayers were washed three times with med 1 and incubated with med 1 supplemented to 100  $\mu\text{M}$  desferrioxamine at 37°C in 5%  $\text{CO}_2$ . One six-well plate per time point (0, 5, 10, and 15 min) was washed once with med 1, solubilized in 0.1% Triton X-100, 0.1 N NaOH, and counted. The mild acid/neutral wash strips cells of uninternalized surface transferrin, thereby ensuring that the efflux of internalized apotransferrin is being followed rather than the sum of the efflux and internalization of surface bound transferrin (McGraw and Maxfield, 1990).

## Cytosolic Acidification Block on Clathrin-Coated Pit Internalization

To measure the increase in surface binding, resulting from the acidification block on internalization, cells grown in six-well clusters were preincubated in 150 mM NaCl, 20 mM HEPES, pH 7.4, 1 mM  $\text{CaCl}_2$ , 5 mM KCl, 1 mM  $\text{MgCl}_2$ , 10 mM glucose (med 1) at 37°C in air for 30 min. Experimental cells were incubated in 10 mM acetic acid, 50 mM MES, pH 5.0, 150 mM NaCl, 1 mM  $\text{CaCl}_2$ , 5 mM KCl, 1 mM  $\text{MgCl}_2$  (med 2) for 15 min. The control cells were incubated in the same pH 5.0 buffer without acetic acid (med 3) for 15 min. The cells were washed and immediately placed on ice. Surface transferrin binding was measured by incubating cells at 4°C with 35 nM  $^{125}\text{I}$ -transferrin for 2 h. Nonspecific binding was determined by including a 100-fold excess of unlabeled transferrin in the incubation buffer.

To directly measure the affect of cytosolic acidification on transferrin internalization cells were preincubated in med 1 for 30 min followed by a 5-min incubation either in med 2 or the control buffer med 3. The buffer was replaced with the appropriate med (2 or 3) supplemented with 35-nM  $^{125}$ I-transferrin. The cells were further incubated for 10 min, rapidly washed with med 1, placed on ice, and then incubated in 0.2 M NaCl, 0.2 M acetic acid (4°C) for 2 min, washed three times with med 1 (4°C), solubilized, and counted. Incubation in 0.2 N NaCl, 0.2 M acetic acid removes all surface bound transferrin, therefore the remaining radioactivity was internalized during the 10 min incubation with  $^{125}$ I-transferrin.

## Results

We have isolated a variant CHO cell line (TRVb cells) that does not express detectable levels of endogenous hamster TR (McGraw et al., 1987). Using this system the behavior of the transfected human TR can be characterized in a background free of functional endogenous receptor (McGraw et al., 1987; McGraw et al., 1988; McGraw and Maxfield, 1990).

The in vitro-constructed mutant receptors examined in this study were transfected into TRVb cells. The correct construction of these mutant receptors was confirmed by dsDNA sequencing of the cDNA used for transfection. A number of independently derived clonal lines were isolated for each of the mutant receptors. We have not observed any significant differences in TR behavior among different clonal lines expressing the same receptor construct. The various clonal lines used in this study all expressed similar numbers of surface TR, so the level of receptor expression is not a complicating factor. Furthermore, we have previously shown that none of the endocytic parameters examined in this study are significantly altered by expressing human TR in TRVb cells at levels between 30,000 and 200,000/cell (McGraw and Maxfield, 1990). All the mutations are restricted to the cytoplasmic domain of the TR and our previous experience indicates that cytoplasmic domain mutations of the TR do

not alter the affinity of the receptor constructs for transferrin (McGraw et al., 1988; McGraw and Maxfield, 1990).

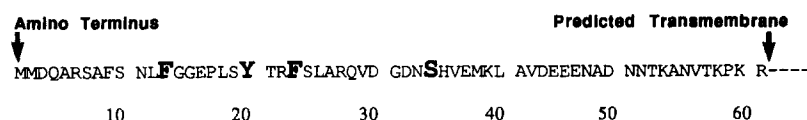
### Phenylalanines at Positions 13 and 23 Are Required for Efficient Internalization

The presence of aromatic amino acids on the cytoplasmic domains of receptors have been shown to influence the rates of internalization. To examine the functional significance of aromatic amino acids, other than the tyrosine, in the cytoplasmic tail of the human TR, we have mutated phenylalanine 13 and phenylalanine 23 (Fig. 1 a) to alanines and characterized the endocytic behavior of these mutant receptors. These phenylalanines were chosen because one (phenylalanine 23) lies within and the other outside the 10-amino acid segment (Fig. 1 a) predicted by deletion analysis to contain the sequences necessary for internalization of the human TR (Jing et al., 1990). Therefore, the expectation was that phenylalanine 23 potentially would be important for internalization whereas phenylalanine 13 potentially would not.

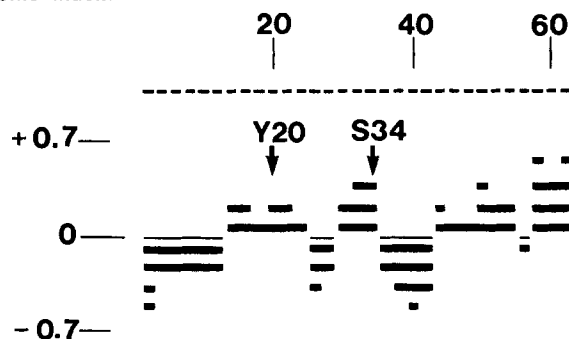
The distribution of the TR between the cell surface and intracellular compartments reflects the ratio of the externalization rate constant ( $k_e$ ; return to cell surface of endocytosed receptor) to the internalization rate constant ( $k_i$ ):  $[TR]_e k_i = [TR]_i k_e$  or  $[TR]_e/[TR]_i = k_e/k_i$ . The wild-type TR expressed in TRVb cells has a surface/internal (S/I) ratio of  $\sim 0.4$ , indicating that the internalization rate constant is  $\sim 2.5$  times that of the externalization rate constant. We have previously shown that substitution of cysteine for tyrosine at position 20 results in a threefold decrease in the internalization rate constant without a detectable effect on the externalization rate (McGraw and Maxfield, 1990). This change is reflected in an S/I ratio of  $\sim 1.2$  for the C20 TR.

As shown in Fig. 2 a, human TR with an alanine substituted for phenylalanine at position 23 has an S/I ratio of

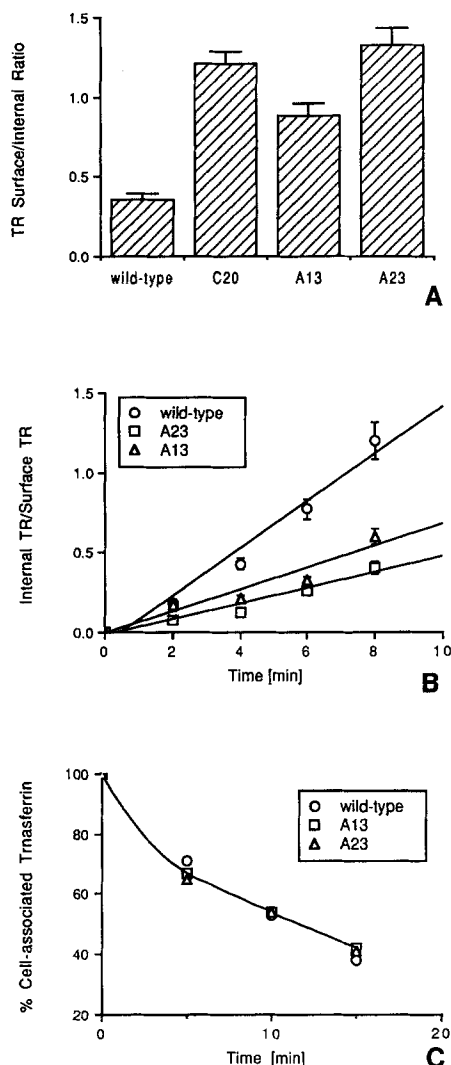
### Cytoplasmic Domain of the Human Transferrin Receptor



### Antigenic Index:



**a** Figure 1. Sequence of the cytoplasmic domain of the human transferrin receptor. (a) The amino acids examined in this study are noted by bold type face. The region predicted by deletion analysis to contain the internalization signal is amino acids 19 through 28 (Jing et al., 1990). The TR sequence has been determined by McClelland et al. (1985). (b) The antigenicity profile of the cytoplasmic domain of the human TR. The algorithm developed by Jameson and Wolf (1988) considers flexibility, hydrophobicity, and potential secondary structure to predicted regions that are likely to be exposed on the surface of a protein. The higher the score the more likely the region is on the surface of the protein. The regions near position 20 and 34 are both predicted to be on the surface of the TR cytoplasmic domain.



**Figure 2.** Endocytic behavior of A13 and A23 TR's. (A) The steady-state TR S/I ratio for the wild-type, and mutant TR's were measured as described previously (McGraw and Maxfield, 1990). The wild-type TR has a tyrosine at position 20 and phenylalanines at positions 13 and 23. The S/I ratio represents the ratio of the endocytic externalization to internalization rate constants. The results presented are the means  $\pm$  SEM of at least six separate determinations. These results demonstrate that the steady-state distribution of the A13 and A23 TR's are affected by these mutations. The measured S/I ratios for all the cell lines examined are presented in Table I. The results for the wild-type and C20 TR's have been reported and have been included for comparison sake (McGraw and Maxfield, 1990). (B) The internalization rate constants were measured as described previously (McGraw and Maxfield, 1990). A plot of the ratio of internalized transferrin to steady-state surface transferrin binding vs. time yields a straight line, whose slope is the internalization rate constant. The data presented are the means  $\pm$  SEM of at least four separate experiments. These results demonstrate that the phenylalanines at positions 13 and 23 are required for efficient internalization of the TR. The measured internalization rate constants for all the cell lines examined are presented in Table I. (C) The externalization rate constants were measured as described previously (McGraw and Maxfield, 1990). The data are graphed as per cent of total cell-associated transferrin remaining vs. time. The values are shown means  $\pm$  SEM of at least four separate determinations. These results demonstrate that these mutations do not effect the recycling of internalized TR back to the cell surface. The exocytic rate constant is determined as the slope of the

$\sim 1.3$  and with an alanine substituted for phenylalanine at position 13 has an S/I ratio of  $\sim 0.9$ . These results demonstrate that the steady-state distribution of TR between the cell surface and internal compartments is perturbed by the substitution of alanine for these phenylalanines and that phenylalanine 23 is as critical as tyrosine 20, whereas phenylalanine 13 is slightly less critical for maintaining efficient internalization.

To more rigorously characterize the behavior of these mutant TR's the internalization and externalization rate constants were directly measured. Using a previously described assay, the TR internalization rate constant can be directly measured (McGraw and Maxfield, 1990). The A23 TR has an internalization rate constant of  $\sim 0.04 \text{ min}^{-1}$  and the A13 TR has an internalization constant of  $\sim 0.06 \text{ min}^{-1}$  (Fig. 2 b). The wild-type and C20 TR have an internalization rate constants of  $\sim 0.13 \text{ min}^{-1}$  and  $\sim 0.04 \text{ min}^{-1}$ , respectively. These rate constants represent the fraction of surface TR internalized per minute.

The externalization rate constants for the mutant TR's were measured and are not significantly different from the wild-type TR (Fig. 2 c). These externalization rate constants represent half-times of 11–12 min for the return to the cell surface of internalized TR. The ratio of the measured endocytic rate constants for the A13 and A23 TR's are in good agreement with the measured steady-state S/I ratios, indicating that the changes in S/I ratio can be completely accounted for by the changes in the internalization rate constants (Table I). These results also confirm our kinetic analysis (internalization and externalization assays) because the measured steady-state S/I distribution agrees with that predicted by the ratio of measured endocytic rate constants.

This kinetic analysis demonstrates that these two phenylalanines play a role in maintaining efficient internalization and that they are required to different degrees. The observations that amino acids near the tyrosine are necessary for maintenance of efficient internalization of the TR and these residues affect internalization to different extents are consistent with similar mutagenic studies of the LDL receptor (Chen et al., 1990).

### *A Second Site Mutation in the Cytoplasmic Tail of the Human TR Converts the Internalization-defective C20 TR to an Efficiently Internalized TR*

To further explore the influence of the amino acid environment surrounding tyrosine 20 on the behavior of the TR, we have created a series of mutant receptors in which the tyrosine has been moved to another position on the cytoplasmic domain. The principal question to be addressed in these experiments is: how does a completely distinct amino acid environment affect the functioning of a tyrosine in maintaining efficient internalization? Our approach to this question was to determine if a tyrosine placed at a different location on the cytoplasmic tail of the human TR could revert the internalization-defective phenotype of the C20 TR. In choosing a site for substitution with a tyrosine we required that (a)

In(cell-associated transferrin) vs. time. The measured externalization rate constants for all the cell lines examined are presented in Table I.

Table I. Endocytic Rate Constants

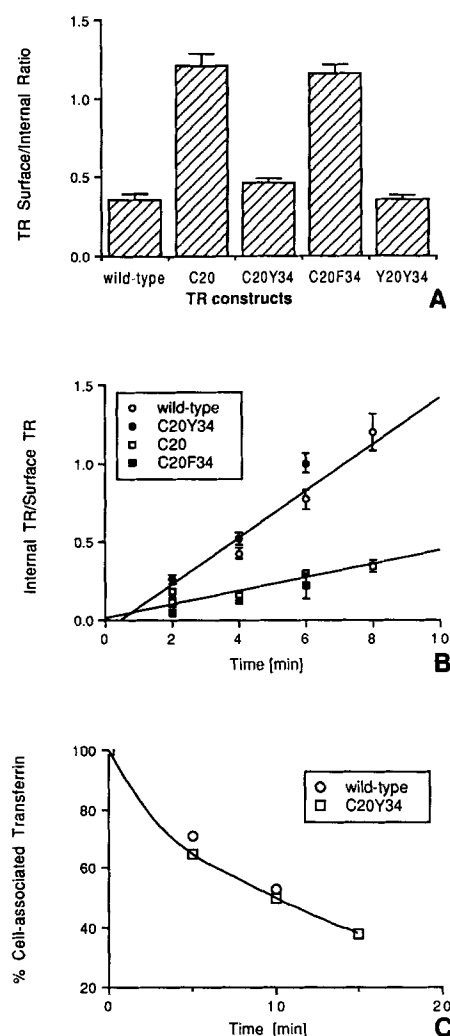
Construct	$k_i^*$ ( $\text{min}^{-1}$ )	$k_e^*$ ( $\text{min}^{-1}$ )	$k_e/k_i^\ddagger$	S/I§	Surface TR expression
Alanine 13	$0.057 \pm .005$	$0.056 \pm .003$	0.98	$0.87 \pm .08$	1.6
Alanine 23	$0.044 \pm .003$	$0.058 \pm .005$	1.31	$1.3 \pm 0.1$	2.1
Cysteine 20/ Tyrosine 34	$0.14 \pm .009$	$0.065 \pm .004$	0.46	$0.48 \pm .04$	0.7

\* Internalization and externalization rate constants, means  $\pm$  SEM were calculated from at least six separate experiments as illustrated in Figs. 2 and 3.

† The TR S/I ratio calculated as the ratio of the measured rate constants.

‡ The measured TR S/I ratio. The values are the means  $\pm$  SEM.

§ Surface TR expressed relative to surface TR expressed in the wild-type cell line used in this study, which expresses  $\sim 120,000$  TR/cell. We have not found any significant alterations in the trafficking of the wild-type TR when expressed in TRVb cells at levels similar to expression as the A13 and A23 TR's.



**Figure 3.** Endocytic behavior of C20Y34, C20F34, and Y20Y34 TR. (A) The steady-state TR S/I ratio for the wild-type and mutant TR's were measured as described previously (McGraw and Maxfield, 1990). The wild-type TR (Y20S34) has a tyrosine at position 20 and a serine at position 34. The results presented are the means  $\pm$  SEM of at least four separate determinations. These results demonstrate that the steady-state distribution of the C20Y34 and Y20Y34 are similar to the wild-type value, whereas the C20F34 S/I ratio is similar to the internalization defective C20 TR. (B) The internalization rate constants were measured as described previously (McGraw and Maxfield, 1990). The data presented are the means  $\pm$  SEM of at least four separate experiments. These results demonstrate that a tyrosine at position 34 reverts the internalization-defective phenotype of the C20 TR, whereas a phenylalanine at posi-

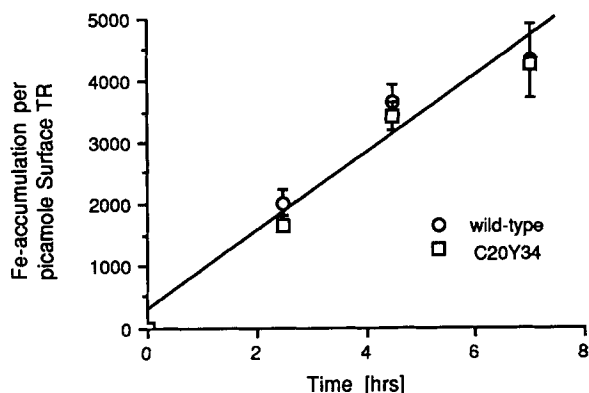
tion 34 does not. The measured internalization rate constants for the C20Y34 TR is presented in Table I. The results for the wild-type and C20 TR's have been reported and have been included for comparison sake (McGraw and Maxfield, 1990). (C) The externalization rate constant for C20Y34 was measured as described previously (McGraw and Maxfield, 1990). The values are shown means  $\pm$  SEM of at least eight separate determinations. These results demonstrate that the C20Y34 TR is returned to the cell surface with identical kinetics as the wild-type and all other mutant receptors examined.

this location be predicted to be on the surface of the protein (thus the tyrosine would be free to interact with other proteins); (b) that the substitution be relatively conservative with respect to protein structure (thereby minimizing potential conformation changes in the cytoplasmic domain). The serine residue at position 34 fulfills these criteria. This serine residue is predicted by antigenicity analysis to be on the surface of the protein (Fig. 1 b), and substitution of serine with a tyrosine is a substitution of a polar noncharged amino acid with another polar noncharged amino acid. Furthermore, it has previously been shown that an alanine residue can be substituted for the serine 34 without altering the behavior of the receptor (Rothenberger et al., 1987).

Three mutant receptors were constructed by oligonucleotide mutagenesis (Fig. 1 a): C20Y34, containing a cysteine substituted for the native tyrosine 20 and a tyrosine for the native serine 34; C20F34, containing a cysteine substituted for the native tyrosine 20 and a phenylalanine for the native serine 34; and Y20Y34, containing the native tyrosine 20 and a tyrosine for the native serine 34.

The endocytic behavior of these double mutant TR's were characterized with the same battery of assays used to characterize the previously discussed mutants. The C20Y34 TR has an S/I ratio of  $\sim 0.5$  which is similar to the S/I ratio of the wild-type receptor (Fig. 3 a). The C20F34 receptor has an S/I ratio of 1.2, which is not significantly different from the internalization-defective C20 TR. The double tyrosine containing TR, Y20Y34, has a wild-type-like S/I ratio of  $\sim 0.4$ .

To demonstrate that the effects were limited to changes in the internalization rate constant, the internalization and externalization rate constants were directly measured. The C20Y34 has an internalization rate constant of  $\sim 0.14 \text{ min}^{-1}$ , which is similar to the wild-type receptor rate constant of  $\sim 0.13 \text{ min}^{-1}$  and significantly higher than the C20 TR internalization rate constant of  $\sim 0.04 \text{ min}^{-1}$  (Fig. 3 b). The C20F34 TR has an internalization rate constant of  $\sim 0.04$



**Figure 4.** Fe accumulation rate. The results presented are from a representative experiment of the assay employed to measure the Fe accumulation rates (McGraw and Maxfield, 1990). Substitution of a tyrosine at position 34 in the C20Y34 TR restores the Fe accumulation rates for C20 to wild-type levels. The Fe accumulation counts are expressed per picomole surface transferrin receptor. The data have been corrected for nonspecific uptake and each point is the average of four measurements  $\pm$  SD.

$\text{min}^{-1}$ . The measured internalization rate constants are consistent with the results of the S/I ratio measurements and they indicate that a tyrosine at position 34 can overcome the internalization defect imposed by substitution of a cysteine for the native tyrosine at position 20, whereas substitution of a phenylalanine at 34 does not influence the internalization rate constant of the C20 receptor.

Endocytosed C20Y34 receptor was returned to the cell surface with kinetics similar to the wild-type and other mutant TR's examined (Fig. 3 c). The ratio of the externalization to internalization rate constants for C20Y34 is in good agreement with the measured steady-state distribution of TR (Table I).

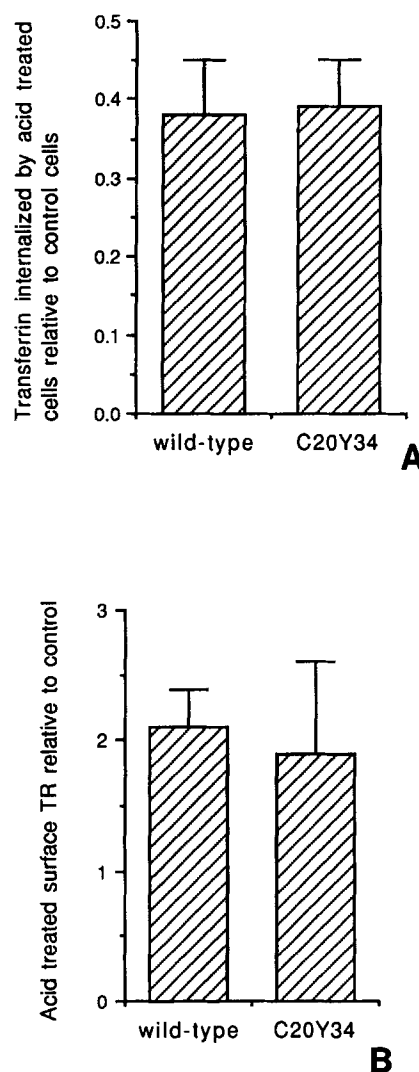
The biological function of the TR is to deliver nutritionally required Fe to cells. To further confirm that the C20Y34 receptor is behaving like the wild-type receptor the rate of Fe accumulation was measured. It has previously been shown that the cells expressing the C20 TR accumulate Fe at  $\sim 60\%$  the rate of cells expressing the wild-type TR (McGraw and Maxfield, 1990). Substitution of tyrosine at position 34 in a background of cysteine 20 restores Fe-accumulation rates to wild-type levels (Fig. 4). The results of the biochemical characterization of the behavior of C20Y34 TR demonstrate that the second-site mutation (tyrosine at position 34) can overcome the internalization-defective phenotype of a C20 TR, resulting in a double-mutant receptor that is trafficked in cells with kinetics identical to the wild-type TR.

### Internalization of C20Y34 TR through Clathrin-coated Pits

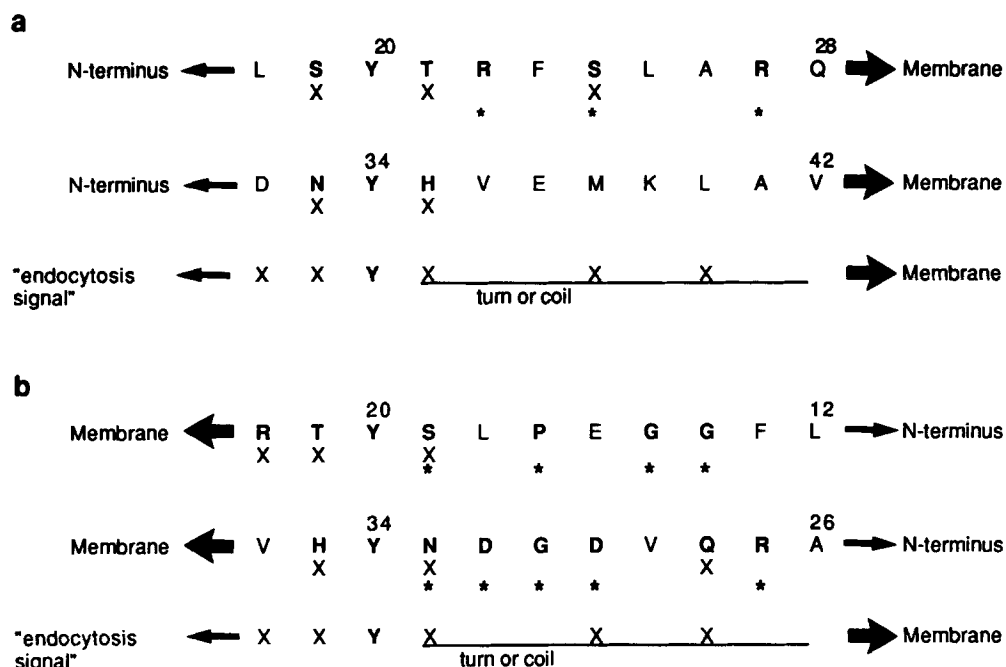
The above biochemical analysis of the trafficking of the C20Y34 TR indicates that this receptor is behaving like the wild-type receptor. The wild-type TR is internalized into cells through clathrin-coated pits (Hopkins and Trowbridge, 1983; Hopkins, 1985). It has been shown that acidification of the cytoplasm to pH  $< 6.5$  results in a dramatic decrease in internalization through clathrin-coated pits (Sandvig et al., 1989). The acidification appears to block the pinching

off of clathrin-coated pits to form endocytic vesicles (Heuser, 1989).

The effect that cytoplasmic acidification has on the internalization of the various mutant TR was examined. Incubation of cells in the presence of 10 mM acetic acid, pH 5.0, buffer decreased the amount of transferrin internalized, by both wild-type and C20Y34 TR's, to  $\sim 40\%$  of control levels (Fig. 5 a). Acidification of the cytosol also resulted in an approximate twofold increase in surface transferrin receptors (Fig. 5 b). The decrease in internalization of transferrin and increase in surface transferrin receptors are consistent with a reduction of clathrin-coated pit internalization without a corresponding block in receptor externalization (Sandvig et



**Figure 5.** Cytosolic acidification increases surface TR. (A) Acidification of the cytosol by incubation in a 10 mM acetic acid, pH 5.0, buffer results in 40% decreases in transferrin internalized by both the wild-type and C20Y34 surface TR's. The data is presented as the ratio of  $^{125}\text{I}$ -transferrin internalized in experimental to control cells. The values presented are averages  $\pm$  SD of three experiments. (B) Acidification of the cytosol results in a twofold increase in surface TR, which is consistent with reduced internalization and continued externalization of the TR's. The values presented are the average  $\pm$  SD of three experiments.



**Figure 6.** Alignment of TR sequences, surrounding positions 20 and 34, to the characteristics of the sequence predicted to comprise the endocytosis signal (Ktistakis et al., 1990): the X indicates a preference for positively charged or polar amino acids at these positions, and the region predicted to be rich in amino acids that are frequently found in random coil or turns is underlined. In *a*, the TR sequences are aligned with the "endocytosis sequence," with the random coil conformation or turn preferred sequences membrane proximal. This is the orientation used by Ktistakis et al. (1990). In *b*, the TR sequences are compared to the endocytosis signal with the reverse orientation, that is, based on the polarity of the peptide chain.

In both panels the polar or positively charged amino acids properly placed with respect to the endocytosis signal are noted below the sequence by an X, and those frequently found in random coil conformations or turns are noted by an \*. The amino acids that align with the model endocytosis signal are in bold type. The position of the amino acids within the TR are noted above the sequences. Tyrosine at position 34, aligned to the endocytosis signal as in *a*, would not be predicted to serve as an internalization signal. However, when the reverse orientation is aligned to the putative endocytosis signal the sequences compare well (*b*). Interestingly, the sequence surrounding the native tyrosine 20 align equally well with the predicted endocytosis signal regardless of orientation. Note in the wild-type TR the amino acid at position 34 is a serine (McClelland et al., 1984).

al., 1989). Furthermore, the approximate twofold increase in surface binding, after 10-min acid treatment, is consistent with the ~60% decrease in internalization. This level of inhibition of internalization through clathrin-coated pits is consistent with what has been reported in other systems (Sandvig et al., 1989). These results indicate that the C20Y34 TR is internalized through clathrin-coated pits and that it can interact with the clathrin-coated pit clustering apparatus.

## Discussion

In this study we have shown that two cytoplasmic phenylalanines of the human TR are required for efficient endocytosis. Substitution of alanines for these phenylalanines affect the efficiency of internalization to different degrees. Substitution at position 23 decreases the internalization rate as much as the previously characterized substitution of a nonaromatic amino acid for the single cytoplasmic tyrosine (McGraw and Maxfield, 1990). Substitution at position 13 results in a significant, albeit more modest, reduction in the internalization rate constant. In these, and our previous studies, we have attempted to delineate the TR internalization signal by identifying the residues, whose replacement results in a reduced rate of internalization. An alternative and complementary approach is to attempt to overcome an internalization-defective phenotype by adding back particular residues. We have found that placement of a tyrosine at position 34 of a C20 TR results in reversion of the internalization-defective phenotype. This second-site reversion appears to be strictly

dependent on tyrosine, as phenylalanine substituted at position 34 does not provide for efficient internalization.

The finding that acidification of the cytosol reduces internalization of the C20Y34 TR indicates that it is being internalized, like the wild-type receptor, through clathrin-coated pits. This result suggests that the C20Y34 TR is not being internalized by an alternative nonclathrin endocytic route and that the placement of a tyrosine at position 34 functionally reconstitutes the TR internalization signal. Thus, we assume that the C20Y34 endocytosis is mediated by association with the same clathrin-adapting protein(s) as the wild-type TR. It is not possible to establish this conclusively because the protein(s) responsible for the concentration of TR in clathrin-coated pits have not been identified. There is evidence from *in vitro* studies, that HA-II clathrin-associated proteins are responsible for clustering of the LDL, IgA, and 275 manose-6-phosphate receptors in clathrin-coated pits (Pearse, 1988; Glickman et al., 1989); however, an association between the TR and the HA-II proteins has not been reported.

The observation that amino acids near the tyrosine are necessary for maintenance of efficient internalization of the TR and that these residues affect internalization to different degrees, are consistent with the results of studies of the LDL receptor (Chen et al., 1990). Along with the previously mentioned NPXY sequence, a phenylalanine five amino acids NH<sub>2</sub>-terminal to the tyrosine has been shown to have a significant effect on the internalization of the LDL receptor, reducing internalization to ~40% of wild-type levels, whereas substitution for the tyrosine reduces internalization to 15% of wild-type level (Chen et al., 1990). Substitution



for TR phenylalanine 13 has a qualitatively similar effect on the TR internalization. This finding is important for understanding the nature of the internalization signal since deletion analysis of the TR has indicated that all the required information is contained within the 10 amino acids between 19 and 28 (Jing et al., 1990). Our results demonstrate that the native clustering signal is influenced by amino acids that lie outside this region. Since this signal is not a linear stretch of amino acids it is likely that identification of all the necessary elements will require a more detailed point mutagenesis study.

Substitution for phenylalanine 23 has as serious an effect on internalization as substitution for the tyrosine 20. In the LDL receptor there is no similarly placed aromatic amino acid with respect to the tyrosine. Interestingly, in the 275-kD mannose-6-phosphate receptor two closely spaced tyrosines have been shown to be required for internalization; however, both tyrosines were simultaneously changed to alanines, so their individual roles in internalization are not known (Lobel et al., 1989).

The efficient internalization of the C20Y34 TR is surprising, because it demonstrates that a tyrosine outside of its native context (that is, position 34 vs. 20) can still provide for efficient internalization. There is precedent, however, for a single amino acid converting a membrane protein from being poorly to efficiently internalized (Lazarovits and Roth, 1988). It has been shown that placement of a tyrosine in the cytoplasmic domain of influenza hemagglutinin, promotes its internalization through clathrin-coated pits. In this case, as shown for position 34 of the TR, internalization is strictly dependent on a tyrosine.

What comprises the clathrin-coated pit clustering (or internalization) signal? To date, all experimental results are consistent with the signal being a specific protein structure, rather than being a linear stretch of amino acids. The requirement for aromatic amino acids, and in some cases, specifically a tyrosine, suggests that the signal might not only require a protein structure but also recognition of a certain chemical property (e.g., aromaticity) or possibly even more specifically a tyrosine, within the context of this structure. An example of protein recognition and sorting based on a physical characteristic of a protein is provided by the signal sequence responsible for transport of proteins into the ER. This signal appears to be based on hydrophobicity, thereby accounting for the rather diverse sequences that can function as signal sequences (von Hiejne, 1985).

Results are somewhat contradictory with respect to the specific role of tyrosines in the internalization signal. On one hand, the behavior of the C20Y34 TR and the tyrosine-containing HA underscore the requirement for a tyrosine. On the other hand, the results that a phenylalanine, when substituted for the native tyrosine, can promote clathrin-coated pit internalization argues against an absolute requirement for tyrosine (Davis et al., 1987; McGaw and Maxfield, 1990). Apparently, the other parts of the recognition signal (e.g., in the TR at least phenylalanines 13 and 23 and in the LDL receptor the asparagine and proline) allow a phenylalanine to function in place of the tyrosine 20. These other elements could allow phenylalanine to function at the native site by either maintaining a specific protein structure and thereby present the phenylalanine in a fashion that can be recognized as well as the tyrosine, or by directly associating with the

clathrin-coated pit protein(s); thereby providing sufficient free energy to compensate for a phenylalanine substituted for the tyrosine. In the former the role of phenylalanines at positions 13 and 23 are as structural elements and would not necessarily be interacting directly with the protein(s) that recognize the signal, whereas in the latter they would be directly recognized. Since a number of different primary amino acid sequences are found surrounding tyrosines required for internalization it seems likely that they are required for maintenance of structure, although these two possibilities cannot be distinguished with the available experimental results.

The amino acids surrounding positions 20 and 34 are not similar. The choice of position 34 as a site for substitution with tyrosine was simply based on the requirement that it would likely be exposed on the surface of the protein and be a relatively conservative change with respect to protein structure. Unfortunately, since there are no reliable means of accurately predicting protein structure, based on primary amino acid sequence, it is not possible to compare the protein structure around the tyrosines at these two different locations.

A model for the endocytosis signal has recently been presented by Roth and co-workers (Ktistakis et al., 1990). This model suggests that the endocytosis signal is comprised of a tyrosine with the surrounding 11 amino acids environment being critical for proper recognition. The two features of the surrounding environment deemed most critical are: (a) That the eight-membrane proximal amino acids are enriched for residues frequently found to break regular structures (propensity for random coil conformation or turns). These residues are glycine, arginine, serine, asparagine, aspartate, and proline (Maxfield and Scheraga, 1976; Chou and Fasman, 1978; Garnier et al., 1978; Gibrat et al., 1987). (b) There is a certain preference for polar or positively charged amino acids at specific positions relative to the tyrosine. In the context of this model, neither of the A13 or A23 mutations in the TR would have been predicted to alter the endocytosis signal. Phenylalanine 13 is not within the putative endocytosis signal region of the TR, and the substitution of an alanine for phenylalanine 23 would not be expected to alter the propensity of the membrane proximal region for random coil conformation or turns.

The region surrounding tyrosine 34 in the C20Y34 double mutant does not fit this paradigm (Fig. 6 a). (A) The region membrane proximal to the tyrosine at 34 does not contain any of the expected amino acids (glycine, arginine, serine, asparagine, aspartate, or proline); (B) A polar or positively charged amino acid is found at only 2 of the 5 positions (asparagine 33, histidine 35). We, however, have shown that a tyrosine at position 34 does function in internalization. The native tyrosine (at position 20) does fulfill the criteria having two arganines and serine in the membrane proximal region and polar or charged amino acids at three of the expected positions (Fig. 6 a).

The endocytosis signal paradigm was developed by aligning the protein sequences based on their orientation with respect to the membrane, rather than the polarity of the peptide chain (Ktistakis et al., 1990). If, however, the TR sequence surrounding position 34 is aligned to the endocytosis signal based on chain polarity (Fig. 6 b), then a tyrosine at 34 would fit the proposed requirements for an internalization



signal. In this alignment five of eight amino acids NH<sub>2</sub>-terminal to the tyrosine favor a random coil conformation or turns, and positively charged or polar amino acids are located at three of the five positions that exhibit the greatest preference for these amino acids, thus satisfying both of the predicted requirements (Fig. 6 b).

Interestingly, the sequence surrounding the native tyrosine 20, when examined in this reverse orientation also aligns to the putative endocytosis signal (Fig. 6, a and b). Thus, it is possible that the orientation of the internalization signal, relative to the membrane, is not critical (i.e., it is not necessary for the random coil/turn to be membrane proximal, as suggested by Ktistakis et al., 1990). If the internalization signal is dependent upon a specific structure, as all available data suggest, its orientation with respect to the membrane might not be critical. Clearly, more detailed mutagenesis studies and structural studies of the cytoplasmic domains of receptors are required to rigorously define the clathrin-coated pit internalization signal.

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