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Human transferrin receptor internalization is partially dependent upon an aromatic amino acid on the cytoplasmic domain

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The objective of this work is to identify the elements of the human transferrin receptor that are involved in receptor internalization, intracellular sorting, and recycling. We have found that an aromatic side chain at position 20 on the cytoplasmic portion of the human transferrin receptor is required for efficient internalization. The wild-type human transferrin receptor has a tyrosine at this position. Replacement of the Tyr-20 with an aromatic amino acid does not alter the rate constant of internalization, whereas substitution with the nonaromatic amino acids serine, leucine, or cysteine reduces the internalization rate constant approximately threefold. These results are consistent with similar studies of other receptor systems that have also documented the requirement for a tyrosine in rapid internalization. The amino terminus of the transferrin receptor is cytoplasmic, with the tyrosine 41 amino acids from the membrane. These two features distinguish the transferrin receptor from the other membrane proteins for which the role of tyrosine in internalization has been examined, because these proteins have the opposite polarity with respect to the membrane and because the tyrosines are located closer to the membrane (within 25 amino acids). The externalization rate for the recycling of the transferrin receptor is not altered by any of these substitutions, demonstrating that the aromatic amino acid internalization signal is not required for the efficient exocytosis of internalized receptor.

Introduction

Receptor-mediated endocytosis is a process through which mammalian cells rapidly and specifically internalize extracellular macromolecules (Goldstein *et al.,* 1985). The majority of receptor-ligand complexes enter cells by clustering in clathrin coated pits. These receptors necessarily contain a signal that distinguishes them from other surface membrane proteins because only a specific subset of membrane proteins is trapped in coated pits (Bretscher *et al.,* 1980; Roth *et al.,* 1986; Miettinen *et al.,* 1989). As a result of this preferential association with coated pits, the receptor proteins are internalized at a rate many times faster than the plasma membrane as a whole (Goldstein *et al.,* 1985).

Studies of in vitro engineered and naturally occurring mutant receptors have demonstrated that the cytoplasmic domains of receptors are required for efficient internalization (Lehrman *et al.,* 1985; Lobel *et al.,* 1989; Mostov *et al.,* 1986; Prywes *et al.,* 1986; McClain *et al.,* 1987; Rothenberger, 1987; Miettinen *et al.,* 1989). The first clue to identifying the internalization signal was the discovery that a single amino acid change in the cytoplasmic domain of the human low density lipoprotein (LDL) receptor, resulting in the substitution of a cysteine for a tyrosine, reduced receptor internalization by approximately fivefold (Davis *et al.,* 1986). Furthermore, it has been shown that the influenza virus hemagglutinin, which is not efficiently internalized, can be converted to an efficiently internalized protein by the substitution of a tyrosine on the short cytoplasmic tail of the protein. (Lazarovits and Roth, 1988). In the three receptors for which it has been examined, high efficiency internalization through coated pits is dependent on an aromatic amino acid on the cytoplasmic domain of the receptor: LDL (Davis *et al.,* 1987); 275 kD mannose 6-phosphate receptor (Lobel *et al.,* 1989); IgA/IgM receptor (Mostov *et al.,* 1988).

The transferrin receptor is a cell surface membrane glycoprotein that mediates cellular iron accumulation via transferrin internalization. The diferric transferrin-receptor complex is internalized through coated pits (Hopkins, 1985), releases bound iron in an acidic endocytic compartment, and is recycled back to the plasma membrane to mediate further rounds of internalization (Hanover and Dickson, 1985). The signals responsible for transferrin receptor internalization and return to the cell surface have

not been identified. In this paper we report that high-efficiency internalization of the human transferrin receptor is dependent on an aromatic amino acid at position 20 of the cytoplasmic domain and that efficient exocytosis is not dependent on this residue. These results are consistent with studies of other receptors and further support the proposal that an aromatic amino acid on the cytoplasmic domain of membrane proteins is involved in efficient internalization.

Results

The human transferrin receptor has a single tyrosine on the 61-amino-acid cytoplasmic domain, which is located 41 amino acids from the inner surface of the plasma membrane (McClelland *et al.,* 1984). To examine the requirement for Tyr-20 in the internalization of the human transferrin receptor, we have, by site-directed mutagenesis, substituted either cysteine, leucine, phenylalanine, serine, or tryptophan for Tyr-20 and characterized the behavior of the altered transferrin receptors stably expressed in TRVb cells, a variant CHO cell line that does not express detectable levels of functional endogenous receptor (McGraw *et al.,* 1987).

Two independently derived single cell colony lines of the Cys-20, Phe-20, and Ser-20 receptor transfectants were isolated by cloning ring colony purification. The duplicate cell lines behaved similarly in all assays. The clonal lines characterized in detail in this paper expressed different numbers of surface transferrin receptors, with Cys-20A expressing ~120 000/cell and Cys-20B ~80 000 per cell, Phe-20C ~60 000/cell, and Ser-20I ~50 000/cell. These clonal cell lines express similar numbers of surface transferrin receptors to the control transfected cell line, TRVb-1, which expresses ~120 000 human wild-type transferrin receptors/cell (McGraw *et al.,* 1987). Although these lines are not identically matched for surface expression, we have not found differences in the behavior of the transfected human receptor in cells expressing between 30 000 and 200 000/cell. Therefore, the different level of receptor expression in these lines is not a complicating factor in the analysis.

Pooled transfectant G-418–resistant cells were also characterized for Phe-20, Ser-20, Trp-20, and Leu-20. Pooled stably transfected cells can be characterized because the recipient cells used in the transfection do not express functional endogenous transferrin receptors (McGraw *et al.,* 1987). Therefore, the behavior of the transfected human transferrin receptor can be characterized in the absence of functional endogenous receptor. The advantage of characterizing a pooled population of cells is that it guards against the possibility that, in an individual colony of cells, the transfected transferrin receptor might behave aberrantly (Zungia and Hood, 1986). In all cases immunofluorescence with a human specific monoclonal antibody to the transferrin receptor (B3/25; Hybridtech, San Diego, CA) that does not cross-react with the hamster receptor confirmed that the transfected cell lines were expressing the human receptor. In the pooled transfectants, between 60% and 80% of the G-418 resistant cells expressed the human transferrin receptor.

As expected, the substitution of Cys-20 on the cytoplasmic tail did not alter the affinity of the receptor for transferrin (not shown). The affinities of the other mutated transferrin receptors were not directly measured. However, we do not expect that these changes in the cytoplasmic domain would alter the extracellular binding site, and high-affinity binding was observed for all of the receptors.

Aromatic amino acid at position 20 of the human TR required for efficient internalization

The internalization rate constant of the transferrin receptor was measured as described in Methods (Figure 1, Table 1). The various transfectants can be grouped into two classes based on internalization rate constants, with the wild-type (Tyr-20), Phe-20, and Trp-20 receptors having internalization rate constants of ~0.11 min^{-1} and the Cys-20, Ser-20, and Leu-20 having rate constants of ~0.04 min^{-1}. The internalization rate constant is the fraction of surface transferrin receptors internalized per minute. Therefore, substitution of Tyr-20 with a nonaromatic amino acid reduces the internalization rate constant nearly threefold. These results indicate that an aromatic amino acid, rather than a more stringent requirement for tyrosine, is required at position 20 of the human transferrin receptor for efficient internalization. Interestingly, the endogenous hamster transferrin receptor (WTB cells) is more rapidly internalized than the wild-type human transferrin receptor expressed in the CHO cell line TRVb, with an internalization rate constant of ~0.2 min^{-1}. At present the sequence of the cytoplasmic domain of the hamster transferrin receptor is not known.

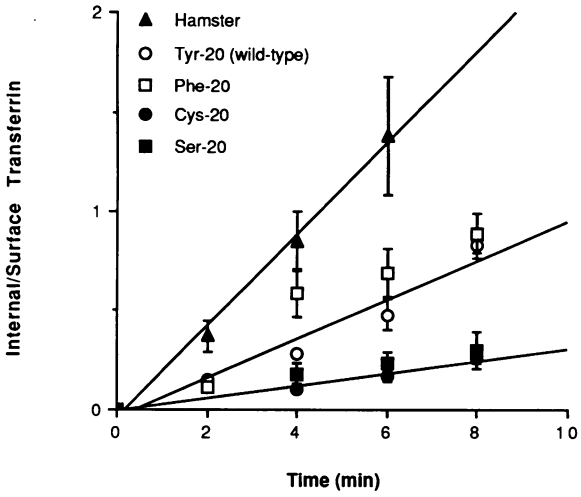

Figure 1. **Internalization rates.** The results presented are from a representative experiment of the internalization assay (described in Methods) employed to measure the internalization rate constants of the altered human transferrin receptors expressed in the CHO cell line TRVb. WTB is the wild-type CHO cell line used in this study. The substitution of a nonaromatic amino acid for Tyr-20 results in about a threefold reduction in the internalization rate constant. The data have been corrected for nonspecific uptake, and each point is the average of four measurements. The data are graphed as a ratio of internalized transferrin/surface transferrin versus time. The values are shown ±SD. The measured internalization rate constants for all the cell lines examined are presented in Table 1.

Table 1. Comparison of internalization rate constants

Transferrin receptor expressed	Internalization rate constant (min^{-1}) ± SE	Percentage of control*
Human Tyr-20 (wild-type)	0.113 ± 0.006	100
Human Phe-20p	0.099 ± 0.008	88
Human Phe-20C	0.123 ± 0.005	108
Human Trp-20p	0.106 ± 0.01	94
Human Cys-20A	0.037 ± 0.004	33
Human Ser-20p	0.039 ± 0.008	35
Human Ser-20I	0.044 ± 0.008	38
Human Leu-20p	0.035 ± 0.01	31
Hamster (WTB cells)	0.225 ± 0.009	200

These values represent the average of at least three separate internalization rate determinations, as illustrated in Figure 1. *Relative to the wild-type human transferrin receptor expressed in TRVb cells. The TRVb-1 (Tyr-20 line), Cys-20A, Phe-20C, and Ser-20I are clonal lines. Phe-20p, Trp-20p, Ser-20p, and Leu-20p are pooled G-418 resistant cell lines. WTB are wild-type CHO cells.

Transferrin receptor exocytic rate is not affected by substitution of nonaromatic amino acid at position 20

Because apotransferrin returns to the cell surface with its receptor and is then rapidly released, it is possible to characterize the rate of return of the transferrin receptor back to the plasma membrane by following the release of apotransferrin from the cell. The exocytic rate constants of the various transferrin receptors were measured as described in Methods. All the human transferrin receptor constructs examined were returned to the cell surface at similar rates, with half-times on the order of 10 min (Figure 2, Table 2). Therefore, the exocytic rate, unlike the internalization rate, is not dependent on an aromatic amino acid at position 20. As was observed for the internalization rate, the hamster transferrin receptor is returned to the cell surface approximately twice as fast as the human transferrin receptor, with a half-time of ~5 min.

Steady-state distribution of transferrin receptors

At steady state, the distribution of transferrin receptors between the cell surface and internal pools reflects the ratio of the exocytic rate constant to the internalization rate constant. Therefore, the ratio of surface transferrin receptor to internal transferrin receptor (S/I ratio) reflects the overall kinetics of transferrin receptor cycling. The S/I ratio was determined as de-

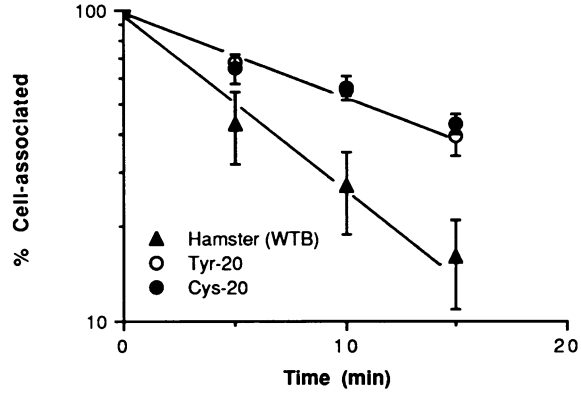

Figure 2. **Exocytic rates.** The results presented are an example of the efflux assay (described in Methods) employed to measure the exocytic rate constants of the altered human transferrin receptors expressed in the CHO cell line TRVb. WTB is the wild-type CHO cell line used in this study. The substitution of a non-aromatic amino acid, cysteine, for Tyr-20 does not alter the exocytic rate of the transferrin receptor. The data have been corrected for nonspecific uptake, and each point is the average of four measurements. The data are graphed as the log of the percentage of total cell-associated transferrin remaining versus time. The values are shown ±SD. The exocytic rate constant is determined as the slope of the ln(cell-associated transferrin) versus time. The measured exocytic rate constants for all the cell lines examined are presented in Table 2.

Table 2. Comparison of exocytic rates

Transferrin receptor expressed	Exocytic rate constant (min^{-1}) ± SD	Percentage of control*
Human Tyr-20 (wild-type)	0.057 ± 0.005	100
Human Phe-20p	0.061 ± 0.02	107
Human Phe-20C	0.058 ± 0.008	102
Human Cys-20A	0.053 ± 0.003	93
Human Ser-20p	0.059 ± 0.02	103
Human Ser-20I	0.062 ± 0.006	108
Hamster (WTB cells)	0.123 ± 0.02	215

These results represent the average of at least three separate determinations of the externalization rate constant, as illustrated in Figure 2. *Relative to the wild-type human transferrin receptor expressed in TRVb cells. The TRVb-1 (Tyr-20) Cys-20A, Phe-20C, and Ser-20I are clonal cell lines. The TRVb cells expressing Phe-20p or Ser-20p are pooled G-418 resistant cell lines. WTB are wild-type CHO cells.

scribed in Methods for wild-type CHO cells (WTB), TRVb cells expressing the wild-type (Tyr-20) human transferrin receptor (TRVb-1 and TRVb-3), and five mutant receptors (Figure 3). The S/I ratio for the wild-type human transferrin receptor is ~0.3. TRVb-3 cells express ~35 000 receptors/cell, about one-third the number of surface transferrin receptors as TRVb-1. Substitution with either of the aromatic amino acids phenylalanine or tryptophan did not dramatically affect the S/I ratio, whereas substitution with the nonaromatic amino acids cysteine, leucine, or serine shifted the S/I ratio to ~1.

The threefold difference in the S/I ratio is consistent with the observed threefold decrease in the internalization rate constant and no alteration in the exocytic rate constant. Although the endogenous hamster receptor is both internalized and recycled more rapidly than the human transferrin receptor, its steady-state distribution is similar to the wild-type human transferrin receptor expressed in CHO cells.

Fe accumulation

Another method for examining the functional behavior of the transferrin receptor is to assay cellular ^{59}Fe accumulation from diferric transferrin. It was predicted that if the only difference between the receptors containing either an aromatic or a nonaromatic amino acid at position 20 is the rate of internalization, then the rate of Fe accumulation should be reduced by the same extent as internalization. The rate of Fe accumulation was measured as described in Methods. The measured rate constants of Fe accumulation per surface receptor for cells expressing the wild-type Tyr-20 transferrin receptor and the Cys-20 transferrin receptor were different by only a factor of 2, rather than the factor of 3 anticipated based on the threefold reduction in the internalization rate constant (Figure 4, Table 3). To be certain that the differences between the anticipated and measured rate constants of Fe accumulation for the Cys-20 transferrin receptor were reproducible, the Fe accumulation rates for the Tyr-20 and Cys-20A cell lines were measured in the same experiment on nine different occasions, and the average ratio of Fe accumulation rates (Tyr-20/Cys-20) was 2.1 ± 0.1 (±SE), whereas the ratio of internalization rates measured on seven separate occasions was (Tyr-20/Cys-20) 2.9 ± 0.1 (±SE). The Fe accumulation rate is not peculiar to the Cys-20A cell line but is characteristic of the Cys-20 receptor, as a second independently derived TRVb line expressing the Cys-20 human transferrin receptor (Cys-20B) accumulates Fe at a rate similar to Cys-20A (Table 3).

As was observed with the internalization rates, the altered receptors can be placed into two groups according to the Fe accumulation, and this classification correlates with the presence of an aromatic amino acid at position 20.

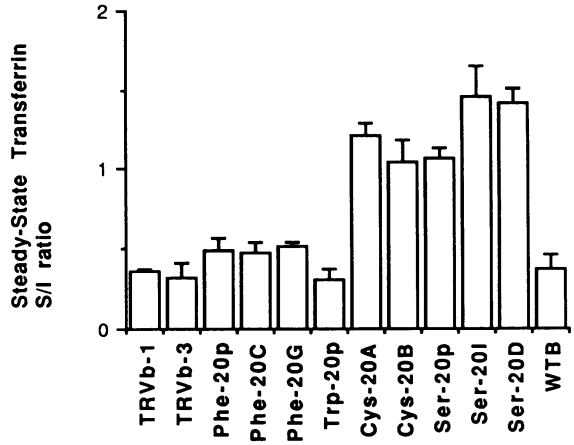

***Figure 3.* The transferrin receptor surface/internal ratio.** The steady-state surface/internal ratio for transferrin was measured as described in Methods for wild-type and altered transferrin receptors expressed in the CHO cell line TRVb. WTB cells are the wild-type CHO cell line used in this study. TRVb-1 and TRVb-3 are two independently derived clonal cell lines expressing the wild-type (Tyr-20) human receptor. Cys-20A, Cys-20B, Phe-20C, Phe-20G, Ser-20D, and Ser-20I are clonal transfectant cell lines. Ser-20p, Leu-20p, Phe-20p, and Trp-20p are pooled G418-resistant transfected cells. Substitution of Tyr-20 with a nonaromatic amino acid shifts the S/I from ~0.3 to 1. Substitution with Phe-20 or Trp-20 does not significantly alter this ratio. The values presented represent the average of at least four separate determinations ± SE.

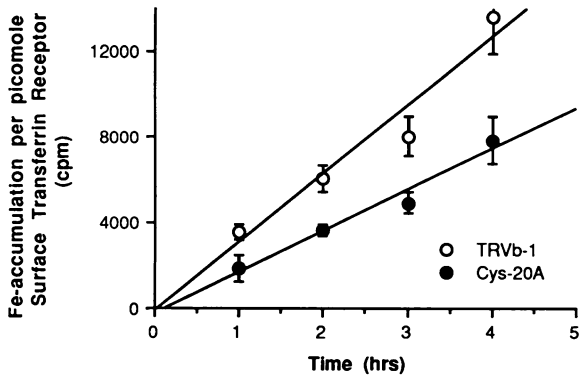

Figure 4. **Fe accumulation rate.** The results presented are from a representative experiment of the assay (described in methods) employed to measure the Fe accumulation rates of the altered human transferrin receptors expressed in the CHO cell line TRVb. Substitution of Tyr-20 with the nonaromatic amino acid cysteine reduced the rate constant of Fe accumulation by twofold. 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 ±SD.

The rate of Fe accumulation in cells expressing Ser-20 receptor is similar to the Cys-20 receptor, and the Phe-20 is similar to the Tyr-20 (Table 3).

The internalization-reduced receptors (nonaromatic amino acid at position 20), having a 70% reduced internalization rate constant but only a 50% reduction in the Fe accumulation rate constant, are more efficient at accumulating Fe from transferrin per internalized receptor than are the rapidly internalized (aromatic amino acid at position 20) receptors. This result suggests that the amino acid at position 20 is also involved in a postinternalization step.

Discussion

We have shown that efficient internalization of the human transferrin receptor is dependent on the presence of an aromatic amino acid on the cytoplasmic domain. The native receptor has a tyrosine at position 20 of the cytoplasmic domain. Replacement of this tyrosine with phenylalanine or tryptophan does not significantly alter the internalization rate constant. However, substitution with serine, leucine, or cysteine reduces internalization by nearly threefold (Table 1).

Our results are consistent with recent studies of other receptors, which have also documented the involvement of a cytoplasmic tyrosine in efficient internalization of the LDL receptor (Davis *et al.,* 1987), the polymeric IgA receptor (Mostov *et al.,* 1988), and the mannose 6-phosphate receptor (Lobel *et al.,* 1989). In each of these cases, replacement of cytoplasmic tyrosine with a nonaromatic amino acid reduced the internalization rate constant by 3- to 10-fold. Furthermore, placement of a tyrosine in the 10-amino-acid-long cytoplasmic domain of the influenza virus hemagglutinin, which normally is not efficiently internalized, causes this protein to be rapidly internalized (Lazarovits and Roth, 1988). These results have supported the assignment of a cytoplasmic tyrosine as an internalization signal. We have shown, as has been shown for the LDL receptor (Davis *et al.,* 1987), that substitution with an aromatic amino acid is sufficient to support efficient internalization, thereby suggesting that it is the aromatic character of the residue at this position that is important rather than specifically a tyrosine.

Each of the other membrane proteins for which the requirement of a tyrosine in efficient internalization has been examined has its carboxy terminus in the cytoplasm. The transferrin receptor has the opposite polarity, with the amino terminus of the protein in the cytoplasm. Our results generalize the aromatic internalization signal to include both types of membrane orientations. Furthermore, the required tyrosine in the other membrane proteins examined is within 25 amino acids of the predicted transmembrane domain, whereas in the transferrin receptor the tyrosine is located 41 amino acids from the membrane. Therefore, if it is required that the tyrosine be located near the membrane (Davis *et al.,* 1987; Lazarovits and Roth, 1988),

Table 3. Comparison of Fe accumulation rates

Transferrin receptor expressed	Fe accumulation rate constant (h^{-1}) ± SE*	Percentage of control†
Human Tyr-20 (wild-type)	3585 ± 358	100
Human Phe-20p	3313 ± 400	92
Human Phe-20C	3807 ± 303	106
Human Cys-20A	1740 ± 245	49
Human Cys-20B	1660 ± 337	46
Human Ser-20p	2100 ± 415	59
Human Ser-20l	2264 ± 260	63

These results represent the average of at least three separate determinations of the Fe accumulation rate constant, as illustrated in Figure 4. *The rate values are expressed as cpm ^{59}Fe-accumulated/picomole surface transferrin receptor/h. †Relative to the wild-type human transferrin receptor expressed in TRVb cells. TRVb-1 (Tyr-20), Cys-20A, Cys-20B, Phe-20C, and Ser-20l are clonal cell lines. Phe-20p and Ser-20p are pooled G-418 resistant transfectant cell lines.

then the cytoplasmic domain of the transferrin receptor must be structured to allow the tyrosine to be close to the membrane.

Current belief is that the tyrosine (or, more aptly, an aromatic amino acid) is involved in the efficient clustering of the receptors in coated pits, and it is this reduced clustering that results in a reduced internalization rate. Supporting this proposal are the observations that the mutant LDL receptor lacking the cytoplasmic tyrosine is not clustered in coated pits (Davis *et al.,* 1987) and that the influenza virus hemagglutinin can be converted from a protein that is excluded from coated pits to a protein concentrated in coated pits by placement of a tyrosine on the cytoplasmic tail (Lazarovits and Roth, 1988). It has also been shown that the binding of the cytoplasmic tails of receptors to the HA-II clathrin-associated proteins is sensitive to the presence of a tyrosine (Pearse 1988, Glickman *et al.,* 1989). Based on these results, we anticipate that transferrin receptors with a nonaromatic amino acid at position 20 do not cluster in coated pits with the same efficiency as the native receptor. In support of this, we have observed by fluorescence microscopy that the mutated transferrin receptors appear diffusely distributed on the plasma membrane (not shown), but the resolution of this technique is not sufficient to rigorously establish reduced clustering.

At present it is not known whether the aromatic side chain is required for maintenance of the structure of the cytoplasmic domain that is recognized for clustering in coated pits or if it is directly involved in the recognition event. The results on mutant influenza virus hemagglutinin would suggest that the aromatic side chain is directly involved in recognition, because the tyrosine is presented in the short, 10-amino-acid sequence. Although it is likely that the aromatic side chain is involved directly in recognition, other aspects of the cytoplasmic domain are probably involved. Supporting this hypothesis, the removal of the transferrin receptor aromatic internalization signal reduces the internalization rate constant threefold, whereas it has previously been shown that a deletion of a large portion (35 of 61 amino acids including the tyrosine) of cytoplasmic domain essentially abolishes transferrin receptor internalization (Rothenberger *et al.,* 1987).

The rate at which the internalized human transferrin receptor is returned to the cell surface is not dependent on an aromatic amino acid at position 20 of the cytoplasmic domain. All the altered transferrin receptors examined in this study were returned to the cell surface with similar rates, demonstrating that the efficiency of recycling back to the cell surface is independent of the rate of internalization. The observation that the hamster transferrin receptor is more rapidly returned to the cell surface suggests that some feature of the receptor can enhance the rate of recycling. Thus, although recycling of membrane proteins back to the cell surface after internalization might be a default pathway, these proteins can be trafficked back to the plasma membrane at different rates. At present there is no information regarding the nature of the signal that determines this rate.

An intriguing observation with regard to the trafficking of the transferrin receptor is that the rate of Fe accumulation is not solely dependent on the receptor internalization and exocytic rates. If the only effect of the nonaromatic amino acid substitution on the trafficking of the transferrin receptor is to reduce the rate constant of internalization, then it would be predicted that the rate constant for cellular Fe accumulation would be reduced to the same extent. However, cells expressing transferrin receptors with nonaromatic amino acids at position 20 accumulate Fe at one-half the rate of the tyrosine- or phenyalanine-containing receptors, rather than at one-third the rate, as would be expected based on the reduced rate of internalization. The Fe accumulation rate constant has been corrected for the number of surface transferrin receptors expressed in the individual cell lines and therefore reflects the rate of Fe accumulation per transferrin receptor. This result suggests that the "aromatic amino acid signal" is involved in a postinternalization event as well.

A possible explanation for this result is that these altered transferrin receptors are spending more time in a cellular compartment that provides efficient release of Fe from transferrin. The available data suggest that, in CHO cells, Fe is released from transferrin in an early acidic endocytic compartment (Yamashiro and Maxfield, 1987). This early compartment is also believed to be the compartment in which recycled proteins are sorted from those destined for lysosomal degradation (Dunn *et al.,* 1989). If the "aromatic internalization signal" is also involved in efficient sequestering of the transferrin receptor in the internal sorting step (analogous to the clustering in coated pits), then the nonaromatic substituted transferrin receptors might spend more time in the acidic compartment where Fe is released from transferrin. This sorting step necessarily is not the rate limiting step in the recycling of transferrin back to the cell surface, because the overall rate is not affected by the substitutions examined in this re-

port. The intracellular steady-state distribution of transferrin receptors suggests that, in CHO cells, a postsorting event is the rate limiting step in recycling, as the receptor accumulates in a postsorting compartment near the Golgi complex (Yamashiro *et al.*, 1984, McGraw *et al.*, 1987). In CHO cells, the sorting compartment, observed by fluorescence microscopy, appears as vesicles in the periphery of the cell (Dunn *et al.*, 1989). The overall steady-state distribution of internalized fluorescein-transferrin is not altered by the nonaromatic amino acid substitution (not shown). However, because the majority of transferrin is concentrated in the para-Golgi recycling compartment, subtle changes in the rate of movement through the sorting compartment would not be detectable by this analysis.

Although it is tempting to speculate that the cytoplasmic aromatic side chain is involved in an intracellular sorting step, it is important to note that the data presented are only suggestive, and confirmation of this effect requires more detailed analysis of the intracellular trafficking of these receptors. This work is presently in progress.

The results of this study extend the previously described "tyrosine internalization signal" to include the human transferrin receptor and demonstrate that the critical feature of this signal is an aromatic side change rather than a more stringent requirement for tyrosine. Furthermore, the results on Fe accumulation suggest that this internalization signal might also be involved in postinternalization events as well.

The difference in recycling kinetics between the hamster and human transferrin receptors suggests that other features of the receptor may be involved in establishing the internalization rate as well as the rate of return to the cell surface. An alternative explanation for the differences between the human and hamster receptors is that the clonal cell line, used in all transfectants, has altered kinetics of recycling. If these differences exist, they would not affect the comparisons among wild-type and altered human receptors transfected into these cells.

After submission of this manuscript, studies on the identification of the internalization signal of the human transferrin receptor expressed in chicken embryo fibroblasts have been reported (Jing *et al.*, 1990). These results are consistent with the requirement for Tyr-20 in efficient internalization and suggest that residues 19–28 comprise the complete internalization signal.

Methods

Cells

The Chinese hamster ovary 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). TRVb-1 cells are a line of TRVb cells that have been stably transfected with a wild-type cDNA clone of the human transferrin receptor (McGraw *et al.*, 1987). The behavior of the wild-type and in vitro mutagenized human transferrin receptors expressed in stably transfected TRVb cells has been previously reported (McGraw *et al.*, 1987, 1988). Stably transfected cells were carried in Ham's F12 media supplemented to 200 μg/ml G418, 14 mM $NaHCO_3$, 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. WTB cells were used as the wild-type CHO cell line (Thompson and Baker, 1973). All cells were grown at 37°C in humidified atmosphere of 5% CO_2 in air. All tissue culture media and supplements were purchased from GIBCO (Grand Island, NY).

Ligands

Human apotransferrin was purchased from Sigma (St. Louis, MO) and further purified by Sephacryl S-300 gel filtration before to use. Diferric transferrin, iodinated diferric transferrin, and ^{59}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 (San Pablo, CA). In each case the cytoplasmic tyrosine residue located at position 20, from the amino-terminus of the human transferrin receptor–predicted sequence (McClelland *et al.*, 1984), was changed to: cysteine, 5′-GGTACATGACAATGG-3′; phenylalanine, 5′-CCGGGTAAATGACAATGG-3′; leucine, 5′-CCGGGTAAGTGACAATGG-3′; serine, 5′-CCGGGTAGA-TGACAATGG-3′; tryptophan, 5′-CCGGGTCCATGACAATGG-3′. The nucleotide changes were confirmed by Sanger sequencing across the mutated site.

Complete human transferrin receptor cDNA clones were reconstructed containing the in vitro altered fragments as previously described (McGraw *et al.*, 1988). The altered receptors were introduced into TRVb cells either by Ca-phosphate precipitation or by Lipofectin (BRL, Bethesda, MD) transfection. In all cases pSV3-Neo was cotransfected into the cells as a dominant selectable marker.

Transferrin receptor recycling assays

Internalization rate. The rate of transferrin internalization was measured using a modification of the In/Sur plot method (Wiley and Cunningham, 1982). Monolayers of cells grown in six-well plates (~10^6 cells/well) were washed and incubated in McCoy's growth salts supplemented to 26 mM $NaHCO_3$ and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) pH 7.4 (med 1) for 15 min at 37°C in 5% CO_2. The cells were washed with med 1 and incubated in med 1 supplemented to 2 mg/ml ovalbumin and 3 μg/ml ^{125}I-Tf (~35 nM) at 37°C in 5% 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 immediately 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 3 times with med 2 (150 mM NaCl, 20 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$)

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 transferrin receptor was determined by incubating a six-well plate of cells with 3 μg/ml ^{125}I-Tf 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 transferrin receptors internalized per minute. For each six-well plate a 200-fold excess of nonradioactive diferric human transferrin was added to two of the wells to measure the nonspecific binding. In general, for all the biochemical assays, nonspecific counts per minute (cpm) were 5% or less of the total.

For our modified In/Sur plot procedure to measure the internalization rate constant accurately, the amount of transferrin-receptor complex on the surface of cells must remain constant over the course of the experiment (Wiley and Cunningham, 1982). Because the transferrin receptor is believed to cycle in the absence of added ligand, this is a reasonable assumption (Hopkins and Trowbridge, 1983; Ajioka and Kaplan, 1986). To demonstrate that the amount of surface transferrin–transferrin receptor complex does not vary over the course of the internalization rate experiments. an internalization rate assay was performed with the substitution of noniodinated transferrin (3 μg/ml) for iodinated-transferrin. After the incubation times of 0, 2, 4, or 6 min, the cells were placed on ice and surface transferrin was removed by treatment with 50 mM MES pH 5.0, 280 mM sucrose, 100 μM desferroxamine (Sigma) at 4°C for 5 min, followed by two 5-min incubations at 4°C in med 1. Surface transferrin binding was then measured by incubating cells at 4°C with 3 μg/ml ^{125}I-transferrin for 2 h. In two separate experiments, surface transferrin binding after pretreatment with 3 μg/ml transferrin varied from the binding to untreated controls by <5%. Thus treatment of cells with 3 μg/ml of transferrin does not alter the number of surface transferrin receptors over the time course of our experiments. A second criterion that needs to be met for the use of the modified In/Sur plot method is that the concentration of transferrin used must be sufficient to saturate surface transferrin receptors rapidly. Surface transferrin binding was measured at 37°C (temperature of internalization rate measurement) by incubating cells, that had been pretreated for 30 min in 10 mM azide 10 mM 2-deoxy-glucose, with 3 μg/ml of ^{125}I-transferrin in 10 mM azide 10 mM 2-deoxy-glucose for 2, 4, 6, or 8 min. Azide and 2-deoxy-glucose are included to block internalization at 37°C. Surface transferrin binding saturated within 2 min.

Externalization rate. The rate at which internalized transferrin is released from cells is used as a measure of the rate at which internalized transferrin receptors return to the cell surface. Monolayers of cells grown in six-well plates were washed with med 1 and incubated for ~1.5 h at 37°C in 5% CO_2 in med 1 supplemented to 3 μg/ml ^{125}I-Tf and 2 mg/ml ovalbumin. 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 μM desferroxamine at 37°C in 5% CO_2. One six-well plate per time point (0, 5, 10, or 15 min) was washed once with med 1, solubilized in 0.1% Triton X-100, 0.1 N NaOH, and counted. The iron chelator, desferroxamine, was included in the efflux incubation to block potential iron reloading of effluxed apotransferrin. 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.

The mild acid/neutral wash has previously been shown to remove surface-bound transferrin from WTB cells (Salzman and Maxfield, 1988). Control experiments were performed to confirm that this treatment is effective in removing surface transferrin from the hamster cells expressing the human transferrin receptor. TRVb-1 cells that had been preincubated in 10 mM azide and 10 mM 2-deoxy-glucose for 30 min at 37°C were incubated for 45 min with 3μg/ml of ^{125}I-transferrin in med 1 with 10 mM azide and 10 mM 2-deoxy-glucose. Under these conditions internalization is inhibited, and surface binding at 37°C can be examined. The cells were then washed three times with 10 mM azide and 10 mM 2-deoxy-gluocse in med 1 to remove unbound ligand. The cells were then incubated at 37°C in the mild acid wash buffer for 2 min, washed three times with med 1 and incubated for 1 min in med 1. The washes were pooled and counted. The cells were then shifted to 4°C and incubated with 0.2 N acetic acid, 0.2 N NaCl (pH ~2) for 2 min. The media were removed and counted. In three separate experiments the mild acid/neutral wash removed 85 ± 1% of the surface counts. The warm, mild acid/neutral wash procedure is preferable to harsh acid or extended 4°C mild acid stripping treatments, even though this procedure leaves a small percentage of receptor-bound transferrin on the surface. The resulting errors in the externalization rate constant are comparable in size to the error of the measurements and thus do not alter our conclusions.

Surface/internal transferrin receptor ratio. At steady state, the ratio of the number of surface transferrin receptors to internal transferrin receptors reflects the ratio of the externalization to internalization rate constants:

$$[TR]_s k_i = [TR]_i k_e \text{ or } [TR]_s/[TR]_i = k_e/k_i$$

where $[TR]_s$ and $[TR]_i$ are surface and internal transferrin receptors, respectively, and k_e and k_i are the exocytic and internalization rate constants, respectively.

The internal pool of transferrin receptors was determined by incubating cells in six-well plates for 1.5 h in med 1 supplemented to 3 μg/ml ^{125}I-Tf. The cells were placed on ice and washed with 0.2 N acetic acid, 0.2 M NaCl for 2 min, washed three times with cold med 2, and solubilized. In parallel dishes the number of surface transferrin receptors was measured by preincubating cells with 3 μg/ml of unlabeled transferrin at 37°C in 5% CO_2 for 1.5 h. The cells were extensively washed with med 1 and incubated at 4°C with 3 μg/ml ^{125}I-Tf for 1.5 h. The dishes were washed with cold med 2, solubilized, and counted. The surface/internal ratio provides a means of assessing the overall recycling behavior (exocytic rate constant/internalization rate constant) of the receptor.

Fe-accumulation. The ability of the various cell lines to accumulate ^{59}Fe from diferric transferrin was assayed as previously described (McGraw *et al.*, 1987). Specific accumulation was determined by subtracting the cell-associated radioactivity in parallel samples in which a 200-fold excess of unlabeled human diferric transferrin had been included in the incubation media. The surface pool of transferrin receptors was measured in parallel dishes by incubating with 3 μg/ml ^{125}I-Tf at 4°C for 1.5 hrs. The cells in one well of each six-well cluster were counted in a hemocytometer after trypsinization. The Fe accumulation counts are expressed as the counts per minute per picomole of surface transferrin receptor.

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