The Transferrin Receptor Cytoplasmic Domain Determines Its Rate of Transport through the Biosynthetic Pathway and Its Susceptibility to Cleavage Early in the Pathway*

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The soluble human transferrin receptor (TfR) found in blood is the result of a proteolytic cleavage occurring in the ectodomain of the receptor close to the transmembrane domain at Arg-100. We have discovered another cleavage site between Gly-91 and Val-92 even closer to the transmembrane domain. Cleavage at Gly-91 differs markedly from the normal cleavage site. It occurs when the entire cytoplasmic portion or the proximal 31 amino acids of the transmembrane domain are deleted. A soluble disulfide-bonded dimer of the TfR is released into the medium in contrast to the cleavage at Arg-100 where a dimer lacking intersubunit disulfide bonds is released. Whereas the cleavage at Arg-100 is generated by cycling through the endosomal system, pulse-chase experiments indicate that cleavage at Gly-91 occurs predominantly during the biosynthesis of the receptor. Pulse-chase analysis of the biosynthesis of mutant TfRs that lack the membrane-proximal cytoplasmic domain show that they exit the endoglycosidase H-sensitive compartment at a slower rate than the wild type TfR. These results suggest that the cytoplasmic domain influences the trafficking of the TfR either by influencing the folding of the ectodomain or by providing a positive signal for its transport through the biosynthetic pathway.

The transferrin receptor (TfR) mediates cellular iron accumulation by binding the iron transport protein transferrin. Iron is released from transferrin in the acidic environment of endosomes, and transferrin and its receptor return to the cell surface where the cycle is repeated 100–200 times during the lifetime of the receptor. The number of TfRs on the cell surface regulates the amount of iron taken into cells. In turn, TfR numbers are regulated at the synthetic level by the stability of its mRNA, which is sensitive to intracellular iron pools. It is regulated at the level of degradation by the release of TfR from the cell by proteolytic cleavage and presumably the lysosomal degradative pathway (1).

The soluble form of the TfR found in blood has been characterized and studied extensively (for review, see Refs. 1 and 2). The TfR is a type II membrane protein with the NH2 terminus in the cytoplasm and the COOH terminus in the ectodomain, and NH2-terminal sequencing of the soluble TfR revealed the cleavage site to be Arg-100 (3). It is released as a homodimer composed of 80-kDa subunits. Under denaturing but nonreducing conditions it migrates as a monomer, indicating a lack of intersubunit disulfide bonds (3, 4). Cleavage occurs during its transit through endocytic compartments after the TfR reaches the cell surface (5). The extent of TfR released into the medium varies with cell type (6). In both rats and humans the amount of soluble TfR in the blood correlates directly with erythropoiesis, indicating that cleavage of the TfR may be the major way of down-regulating the TfR during the differentiation of red blood cell precursors (2, 7).

While examining the trafficking of various mutated TfRs, we discovered a different cleavage site in the ectodomain of the TfR which results in the release of a soluble disulfide-bonded dimer of the TfR. This soluble TfR was sequenced, and the cleavage site was determined to be after Gly-91. Cleavage at Gly-91 occurs in TfRs in which 20 amino acids are deleted in the cytoplasmic domain of the TfR proximal to the membrane (amino acids 29–59). No detectable cleavage of wild type TfR at Gly-91 is detected. We present evidence that the protease that cleaves the TfR at Gly-91 is a resident of the early biosynthetic pathway, further distinguishing this cleavage from the previously characterized endosomal cleavage at Arg-100. Importantly, the extent of cleavage at Gly-91 correlates with the transit rate of the TfR through the ER-Golgi compartments. The longer the TfR spends in the endo H-sensitive compartment (e.g. ER(eis)-Golgi), the greater the extent of cleavage at Gly-91. These results imply that the region of the cytoplasmic domain of the TfR proximal to the membrane is required for rapid transit through the early biosynthetic compartments. The deletion of amino acids 29–59 increases cleavage of the TfR by slowing its transit through the early compartments, thereby increasing the time the TfR is in the compartment containing the protease.

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¶ The abbreviations used are: TfR, transferrin receptor; ER, endoplasmic reticulum; endo H, endo-β-N-acetylglucosaminidase H (EC 3.2.1.96); PCR, polymerase chain reaction; ECL, enhanced chemiluminescence; PAGE, polyacrylamide gel electrophoresis; O-linked, serine/threonine-linked; VSV-G, vesicular stomatitis virus G.
The fragment was gel purified, digested with and the two outside primers, to create a full-length mutated fragment. Fragments were gel purified by electroelution, ethanol precipitated, and created two overlapping fragments, one containing the mutation. These units of Vent polymerase, from an outside primer to an inside primer were 5'-9DH5 ligated into the pcDNA3 TfR plasmid, which had been digested with complete medium and incubated at 37 °C, 5% CO₂. At the indicated times with [35S]Met/Cys in medium lacking Met as described previously (18). After labeling they were washed twice with Triton X-100. The TfR was isolated from the lysates and media with 100 μl × Laemmli buffer and subjected to SDS-PAGE under reducing conditions for media. The positions of reduced high molecular weight markers (Sigma) are indicated. Samples were transferred to nitrocellulose and developed using sheep anti-TfR (1:10,000); horseradish peroxidase-conjugated swine anti-goat (1:10,000), and ECL reagent. The positions of monomer TfR (M) and disulfide-bonded dimer TfR (D) are indicated.

Characterization of Two Juxtamembrane Cleavage Sites in the Ectodomain of the TfR—We have shown previously that the soluble TfR found in blood is generated by cleavage of the wild type TfR at Arg-100 by an unidentified protease (3). The soluble TfR fragment migrates as a monomer on nonreducing SDS-PAGE under reducing conditions for media. The positions of reduced high molecular weight markers (Sigma) are indicated. Samples were transferred to nitrocellulose and developed using sheep anti-TfR (1:10,000); horseradish peroxidase-conjugated swine anti-goat (1:10,000), and ECL reagent. The positions of monomer TfR (M) and disulfide-bonded dimer TfR (D) are indicated.

Cell Culture, Transfection, and Metabolic Labeling—TRVb cells, a Chinese hamster ovary cell line deficient in endogenous TfR (8), were grown in Ham’s F-12 medium (Sigma) with 2 g/liter glucose and 5% fetal bovine serum or in HyQ CCM5 serum-free medium (HyClone, Logan, UT). TRVb cells were transfected with the DNA-encoding mutant transferrin receptors P59A TfR and ∆29–59 TfR in the expression plasmid pcDNA3 (Invitrogen, San Diego) by the calcium phosphate method (9) and selected for resistance to Geneticin as described previously (10). TRVb cells expressing the C29A23 TfR, the ∆3–28 TfR, the ∆3–59 TfR, and the T104D TfR were generated previously (5, 8, 10–13). Cells were metabolically labeled with [35S]Met/Cys (Trans[35S]-label, ICN, Irvine, CA) or [3H]palmitate (NEN Life Science Products) in Ham’s F-12 Met/Cys-deficient medium (Life Technologies, Inc.) with 5% fetal bovine serum.

Mutant Transferrin Receptors—The P59A TfR and the ∆29–59 TfR mutants were made by PCR overlap extension (14). The TfR cDNA in pcDTR1 (15) was cut out with EcoRV and XbaI, eliminating the 5’- and 3’-noncoding regions, and was inserted into pcDNA3, in which the HindIII site in the multiple cloning site was destroyed by digestion with HindIII and filled in with Klenow DNA polymerase. PCR mutagenesis was carried out using the outside primers to the ‘‘T7’’ promoter (5’-TAA TAC GAC TCA TCA TAG GG-3’) and to nucleotides 975–995 of the TfR (5’-ATG TCC AAA GAA TGA AAGTTC-3’). The inside, overlapping, and mutagenic primers for creating the P59A TfR were 5’-AGG GCC AAT GTC ACA AAA GCA AAA AGG TGT AGT GGA-3’ and 5’-TTT TGG CCG AGC CAG GCT-3’. For creating the ∆29–59 TfR they were 5’-CGC CTG CCG CAG AAA AAG TGT AGT GGA-3’ and 5’-TTG CCG AGC CAG GCT-3’. The first round of PCRs, using 3 units of Vent polymerase, from an outside primer to an inside primer created two overlapping fragments, one containing the mutation. These fragments were gel purified by electroelution, ethanol precipitated, and resuspended for the second round of PCR, with 3 units of Taq polymerase (Life Technologies, Inc.), the products from the first PCR round, and the two outside primers, to create a full-length mutated fragment. The fragment was then gel purified, digested with EcoRV and HindIII, and ligated into the pcDNA3 TfR plasmid, which had been digested with EcoRV and HindIII. The resulting plasmids were transfected into DH5α bacteria, and colonies were screened for the correct plasmid, which was then sequenced along the entire length that had been subjected to PCR, about 900 nucleotides. No errors were found. The T104D∆3–59 was made by loop-out mutagenesis of the T104D construct using the Amersham site-directed mutagenesis kit and the oligonucleotide 5’-CAG TTC AGA ATG ATG AAA AGG TGT AGT GGA-3’.

Gel Electrophoresis—Samples were processed for electrophoresis and Western blots as described previously (16). Western blots were developed by incubation with a sheep anti-TfR serum (16) (1:10,000), horseradish peroxidase-conjugated swine anti-sheep (1:10,000) and visualized by enhanced chemiluminescence ECL (Amersham Pharmacia Biotech).

Purification of Soluble TfR and Amino Acid Sequencing—Soluble TfR was purified from 5.5 liters of conditioned medium from TRVb cells expressing the ∆3–59 TfR using a 5-ml transferrin-agarose column as described previously (10). Amino acid sequencing was carried out as described previously (10). Samples were subjected to SDS-PAGE and blotted onto polyvinylidene difluoride, which was subsequently Coomassie stained and destained. Protein bands were excised and subjected to automated Edman degradation sequence analysis in either an Applied Biosystems Inc. 476A or 477A system using standard sequencing cycles. Amino acid determinations were made by visual inspection of resulting chromatograms.

Biotinylation of Cell Surface Proteins—Cells (35-mm dishes) were washed with phosphate-buffered saline and cooled on ice. They were incubated with 1 ml of 3.4 mM sulfosuccinimidyl biotin (Pierce) dissolved in phosphate-buffered saline just before use to label cell surface proteins. After 30 min on ice they were washed three times with medium and incubated in 2 ml of medium overnight (12–17 h) at 37 °C in 5% CO₂. The medium was collected, and the cells were washed and lysed with 1 ml of 50 mM Tris, 5 mM EDTA, 150 mM NaCl, pH 7.5, 1% Triton X-100. The lysate was isolated from the lysates and media by immunoprecipitation with 2 × Laemmli buffer (17) lacking reducing agents. Samples were subjected to SDS-PAGE under nonreducing conditions.

Pulse-Chase with [35S]Met/Cys—Cells (35-mm dishes) were pulsed for the indicated times with [35S]Met/Cys in medium lacking Met as described previously (18). After labeling they were washed twice with complete medium and incubated at 37 °C, 5% CO₂. At the indicated times the medium was collected, and the cells were washed twice with phosphate-buffered saline and lysed with 0.05 M Tris-Cl, 0.15 M NaCl, 5 mM EDTA, pH 7.5, 1% Triton X-100. The lysate was isolated from the medium of cells expressing the wild type TfR (Fig. 1). This large increase was unexpected because cleavage of the wild type TfR at Arg-100 occurs in an endosomal compartment following internalization from the plasma membrane, and the ∆3–59 TfR is internalized significantly more slowly than the wild type TfR (11, 19–21).

A characteristic of the cleavage at Arg-100 is that it is enhanced by elimination of the O-linked oligosaccharide at Thr-104 (T104D) (6, 10). To determine the effect of O-linked glycosylation at Thr-104 on cleavage of the ∆3–59 TfR we
The TfR Cytoplasmic Domain Affects Ectodomain Cleavage

Fig. 2. Cleavage site of the disulfide-bonded TfR. Panel A, amino acid sequence of the first six amino acids on the NH2 terminus of the soluble Δ3–59 TfR reveals a novel cleavage site. The major site is at Gly-91 (80%). A minor site is at Cys-89 (20%). Panel B, amino acid sequence of the NH2 terminus of the TfR including the cytoplastic (amino acids 1–61), transmembrane (amino acids 62–89), and a portion of the ectodomain of the TfR proximal to the transmembrane domain. The novel cleavage site, the cleavage site found in serum, and the O-linked glycosylation site are all in the ectodomain (amino acids 90–780).

constructed a Δ3–59 TfR containing a mutation of Thr-104 to Asp-104 (10) and determined the effect of this mutation on the formation of soluble TfR. The amount of soluble TfR generated from Δ3–59 TfR is greater than that generated from cells expressing the T104D TfR, and elimination of the O-linked glycosylation site does not result in enhanced release of soluble TfR from the Δ3–59 TfR (medium: Δ3–59 and T104DΔ3–59; Fig. 1). Therefore, either the truncated form of the TfR does not contain an O-linked oligosaccharide at this site, or the cleavage at the alternate site is not sensitive to glycosylation (see below).

The soluble TfR generated from the Δ3–59 TfR is heterogeneous on nonreducing gels, and the majority of the released TfR contains at least one intersubunit disulfide bond. In contrast, the soluble TfRs generated from either the wild type TfR or the T104D TfR show no appreciable soluble disulfide-bonded dimer (Fig. 1). Two intersubunit disulfide bonds have been identified in the TfR at Cys-89 and Cys-89 (22, 23). Thus, the new cleavage site had to be between Cys-89 and the putative transmembrane domain starting at Cys-89. To determine the cleavage site, the soluble TfR generated from Δ3–59 TfR was isolated from the medium of transfected cells. NH2-terminal sequencing of the first six amino acids of the soluble disulfide-linked TfR dimer indicates that the major cleavage site is between Gly-91 and Val-92 (~90%) with a minor cleavage site between Cys-89 and Lys-90 (~10%) (Fig. 2). Sequencing eight amino acids of the soluble monomer derived from Δ3–59 TfR on nonreducing SDS gels indicates that the cleavage occurs at Arg-100. Lack of sequencing at Thr-104 is consistent with an O-linked oligosaccharide at this site, suggesting that the Δ3–59 TfR contains an O-linked glycosylation at Thr-104 (24, 25). Under reducing conditions, the mobility of the dimer shifts to 80 kDa. The mobilities of the soluble TfRs cleaved at either Gly-91 or Arg-100 under reducing conditions were indistinguishable from one another (results not shown). Because this cleavage site at Gly-91 is different from the one identified previously we sought to characterize where in the cell the cleavage was occurring and why eliminating the cytoplasmic domain of the TfR resulted in the novel cleavage in its ectodomain.

Cleavage of the TfR at Gly-91 Is Not Correlated with Time Spent on the Cell Surface—Previous studies indicated that cleavage of the TfR at Arg-100 occurs when the TfR cycles through the endocytic compartments (5). The TfR contains the endocytic sequence VTFR in the cytoplasmic domain at amino acids 20–23. The Δ3–59 TfR is defective in endocytosis and accumulates on the cell surface (13, 19, 26). Therefore we wanted to test whether the Gly-91 cleavage was occurring at the plasma membrane because cell surface metalloproteases such as TACE have been implicated in the cleavage of an increasing number of proteins such as the tumor necrosis factor α, receptors, and cell adhesion molecules (27–30). Mutation of two critical amino acids, Tyr-20 → Cys and Phe-23 → Ala (C20A23 TfR), or deletion of the endocytic signal (Δ3–28 TfR) results in TfRs equally as defective in endocytosis as the Δ3–59 TfR (11, 19, 20). Thus if cleavage was on the cell surface then these cytoplasmic mutations should result in the release of a soluble disulfide-linked TfR dimer into the medium. No detectable amounts of cleaved TfR above that of the wild type TfR could be detected in the medium even when large amounts of medium were collected and the blots were exposed longer than normal (Fig. 3). These results indicate that a cell surface protease was not responsible for the cleavage of the TfR at Gly-91. They differ from the generation of the soluble TfR cleaved at Arg-100. In the latter case, elimination of the endocytic signal resulted in less soluble TfR being produced (5). Because cleavage at Gly-91 is seen when the entire cytoplasmic domain is missing and not when the region from amino acids 3–28 is missing, this suggests that the region 29–59 may be important for generating the soluble disulfide-linked TfR.

Cleavage of the TfR at Gly-91 Is Potentiating by Deletion of the Juxtamembrane Region of the Cytoplasmic Domain—The TfR with a deletion in the juxtamembrane region was generated to determine whether this sequence is important in the generation of the soluble disulfide-linked TfR dimer. A soluble TfR containing intersubunit disulfide bonds is generated from Δ29–59 TfR (Fig. 4). This process is not as efficient as in the Δ3–59 TfR (Fig. 3). The cleavage of the TfR missing the NH2-terminal 26 amino acids in the cytoplasmic domain (Δ3–28 TfR) was compared with the cleavage of the TfR missing the 31 transmembrane-proximal amino acids (Δ29–59 TfR) (Figs. 3 and 4). The Δ3–59 TfR produces a greater amount of soluble disulfide-bonded dimer than does the Δ29–59 TfR. Because the Δ29–59 TfR and the Δ3–28 TfR have similarly sized cytoplasmic domains, the composition of the cytoplasmic domain is likely to be more important than the length of the cytoplasmic

FIG. 2. Cleavage site of the disulfide-bonded TfR. Panel A, amino acid sequence of the first six amino acids on the NH2 terminus of the soluble Δ3–59 TfR reveals a novel cleavage site. The major site is at Gly-91 (80%). A minor site is at Cys-89 (20%). Panel B, amino acid sequence of the NH2 terminus of the TfR including the cytoplastic (amino acids 1–61), transmembrane (amino acids 62–89), and a portion of the ectodomain of the TfR proximal to the transmembrane domain. The novel cleavage site, the cleavage site found in serum, and the O-linked glycosylation site are all in the ectodomain (amino acids 90–780).

FIG. 3. Generation of the disulfide-bonded soluble TfR does not depend on endocytosis. Cells expressing wild type TfR (WT) or TfRs lacking the endocytic internalization signal (C20A23), the portion of the cytoplasmic domain distal to the transmembrane domain (Δ3–28), or lacking the cytoplasmic domain (Δ3–59) were plated onto 35-mm dishes. Cells and medium were harvested 4 days after plating. The TfR was isolated from the medium of 600 times more cells with transferrin-agarose than the cell, and lysates were run in lanes to visualize the soluble TfR more clearly. Samples were eluted under nonreducing conditions in 2 × Laemmli buffer and subjected to SDS-PAGE under nonreducing conditions. Samples were transferred to nitrocellulose and developed using sheep anti-TfR (1:10,000), horseradish peroxidase-conjugated swine anti-goat (1:10,000), and ECL reagent. The positions of monomer TfR (M) and disulfide-bonded dimer TfR (D) are indicated. Panel A, 90-s exposure and panel B, 6-s exposure of the soluble TfR isolated from the medium from cells transfected with WT, C20A23, and T104D.
domain in regard to the production of this form of the soluble TfR.

Mutational analysis of residues around a variety of membrane proteins indicates that the protease(s) cleave at a specific distance from the membrane in addition to having amino acid specificity (for review, see Ref. 31). The juxtamembrane domain of the cytoplasmic domain of the TfR might have residues that could anchor the TfR in the membrane at a fixed position relative to the membrane. Altering the amino acids by truncation of this portion of the cytoplasmic domain, as is the case with Δ3–59 TfR and Δ29–59 TfR, could result in a shift of the TfR in the membrane, making it more susceptible to proteolytic cleavage at a new site. One explanation for the cleavage at Gly-91 could be that an α-helix breaking Pro at position 59 influences the position of the TfR in the membrane. However, mutation of Pro-59 to an α-helix permissive Ala had no effect on the cleavage of the TfR (Fig. 4). Another possibility concerns the addition of palmitic acid to the TfR. The covalent modification of proteins with palmitic acid contributes to the anchoring of proteins in membranes (32). The wild type TfR is normally palmitoylated at Cys-61 and perhaps Cys-67 (22, 23).

The truncated Δ5–59 TfR could lack palmitate, and therefore the transmembrane region of the TfR may not be anchored in the membrane properly. Cells containing either wild type or Δ3–59 TfR were labeled with either [3H]palmitate or [35S]Met/Cys. Approximately equal numbers of TfRs were immunoprecipitated and the relative amounts of [3H]palmitate incorporated into the TfRs were visually compared with the amount of [35S]Met/Cys incorporated (Fig. 5). Both the wild type and Δ3–59 TfR contain the same number of methionine and cysteine residues, so such a comparison is valid. Significantly more [3H]palmitate was incorporated into the truncated Δ3–59 TfR than into the wild type TfR, indicating that enhanced cleavage of Δ3–59 is not caused by reduced palmitoylation. Palmitoylation occurs at the plasma membrane (32). Our results are consistent with this observation because a greater proportion of Δ3–59 TfR than wild type TfR is at the plasma membrane.

Cleavage of the TfR at Gly-91 Occurs during the Biosynthesis of the TfR—Earlier studies indicated that generation of the soluble TfR by cleavage at Arg-100 occurred after the TfR had reached the plasma membrane and while it was cycling through endocytic compartments in the cell (5). Two methods were used to determine that most of the soluble TfR cleaved at Gly-91 is generated during the biosynthesis of the TfR before it reaches the cell surface. In one set of experiments cells were labeled with [35S]Met/Cys for 1 h, washed, and chased for up to 24 h. Nonreducing SDS-PAGE analysis of newly synthesized Δ3–59 TfR indicates that the soluble receptor that is cleaved at Gly-91 (dimer on nonreducing SDS gels) is generated within the first 2 h of its synthesis, and very little additional dimer is generated with a longer chase. The cleavage at Arg-100 (monomer on nonreducing SDS gels) takes place continuously and linearly during the lifetime of the TfR (Fig. 6).

To confirm these conclusions, cell surface proteins were biotinylated at 4 °C. The cleaved TfR was isolated from medium 24 h after biotinylation, and the cleavage site and extent of cleavage were analyzed by nonreducing SDS-PAGE. Cleaved biotinylated TfR was detected by horseradish peroxidase-conjugated swine anti-goat (1:10,000), and ECL reagent.

![Fig. 4](Image 66x610 to 280x729)

**Fig. 4.** Cleavage of the TfR is enhanced in the TfRs missing the cytoplasmic portion proximal to the transmembrane domain. Cells expressing wild type TfR (WT) or TfRs lacking the cytoplasmic domain (Δ3–59), the Pro close to the transmembrane region (P59A) or lacking the portion of the cytoplasmic domain (Δ29–59) proximal to the transmembrane domain were plated onto 35-mm dishes. Cells and medium were harvested 4 days after plating. The TfR was isolated from the medium of 10 times more cells than the lysates to visualize the soluble TfR more clearly. Samples were eluted under nonreducing conditions with 2 × Laemmli buffer and subjected to SDS-PAGE under nonreducing conditions. The positions of reduced high molecular weight markers (Sigma) are indicated. Samples were transferred to nitrocellulose and developed using sheep anti-TfR (1:10,000), horseradish peroxidase-conjugated swine anti-goat (1:10,000), and ECL reagent.

![Fig. 5](Image 363x636 to 499x729)

**Fig. 5.** Cleavage is not the result of a lack of palmitate anchoring the Δ3–59 TfR in the membrane. Cells expressing the wild type TfR (WT) or the Δ3–59 TfR lacking the cytoplasmic domain were labeled overnight with either [35S]Met/Cys (5 μCi) or with [3H]palmitate (100 μCi). Lysates were immunoprecipitated with anti-TfR serum. Samples were subjected to SDS-PAGE (8% acrylamide) under reducing conditions and to autoradiography.
the loss of complete sensitivity to endo H can be followed. The wild type and D3–28 TfRs become partially endo H-insensitive within 30–60 min of the chase period (Fig. 8A). In contrast, the D29–59 and the D3–59 TfRs are significantly retarded in their loss of endo H sensitivity, indicating that they pass through the early portion of the biosynthetic pathway slower than the wild type or D3–29 TfRs. Quantitation of the loss of endo H sensitivity confirms these observations (Fig. 8B). Transit through the biosynthetic pathway can be affected by the level of expression of transfected proteins. Overexpressed proteins can saturate the biosynthetic pathway and slow their transit. All of the transfected cell lines expressed within a factor of three the same amounts of TfR. The highest expression level was that of the wild type TfR. This level was within the same range of concentrations of wild type TfR measured in human cell lines (1–3 \times 10^5 surface TfR/cell). Therefore, the biosynthetic pathway should not be saturated in these cell lines.

DISCUSSION

Soluble counterparts of a variety of cell surface proteins have been found in serum. Among these are transforming growth factor α (34), kit receptor ligands (35), interleukin-2 receptor (36, 37), mannose 6-phosphate receptor (38), platelet-derived growth factor receptor (39), and the transferrin receptor (40). Cleavage occurs, depending on the protein, at various cellular

Fig. 6. Accumulation of the disulfide-linked soluble TfR during biosynthesis of the TfR. Cells expressing the Δ3–59 TfR, which lacks the cytoplasmic domain, were labeled with 25 μCi of [35S]Met/Cys either overnight (ON) or for 1 h. At time 0 they were washed twice with medium and incubated at 37°C in 5% CO₂ in complete medium. The medium from the cells was harvested at the indicated times, and the TfR was isolated with transferrin-agarose. The samples were subjected to SDS-PAGE (6% acrylamide) under nonreducing conditions and to autoradiography. The positions of monomer TfR (M) and disulfide-bonded dimer TfR (D) are indicated.

Fig. 7. Biotinylation of cell surface TfR. Cells expressing the TfRs either lacking the cytoplasmic domain (Δ3–59) or lacking the O-linked glycosylation site were biotinylated on ice to label only cell surface proteins as described under “Materials and Methods.” They were washed and incubated in complete medium overnight at 37°C in 5% CO₂. The medium was collected and the cells lysed. TfR was isolated with transferrin-agarose. Sample were subjected to SDS-PAGE under nonreducing conditions, transferred to nitrocellulose, and detected with horseradish peroxidase-conjugated streptavidin (1:8,000) and ECL.

Fig. 8. Pulse-chase and endo H treatment of the wild type TfR and the mutant TfRs with truncated cytoplasmic domains. Cells expressing the wild type TfR (WT), the TfR lacking the cytoplasmic domain distal to the transmembrane domain (Δ3–28), the TfR lacking the cytoplasmic domain proximal to the transmembrane domain (Δ29–59) or the TfR lacking the cytoplasmic domain (3–59) were pulsed with 100 μCi of [35S]Met/Cys for 10 min in Met-free medium containing 5% fetal bovine serum. They were then washed and chased for up to 120 min. At each time point, cells were solubilized and the TfR isolated with anti-TfR serum and Staphylococcus aureus. The immunoprecipitate was divided in half, resuspended in pH 6.0 buffer, and digested (1) or mock digested (2) with endo H (New England Biolabs) for 2 hr at 37°C according to the manufacturer’s directions. Panel A, samples were subjected to SDS-PAGE (8% acrylamide) and autoradiography. The position of the band that is totally sensitive to endo H (~80 kDa) is marked on the right portion of the gel (arrows). Panel B, the loss of endo H sensitivity of the TfRs at each time point was quantitated by scanning the autoradiographs and integrating the densities using NIH image 1.57. Films were preflashed, and densities were within the linear range of the film.
locations. For example, cleavage of transforming growth factor α occurs at the plasma membrane (41), the TfR is cleaved within an endocytic compartment (5), and the H2α subunit of the asialoglycoprotein receptor is cleaved during transit through the biosynthetic pathways (42). The physiological functions of the soluble receptors vary. In the case of signaling receptors (e.g., interleukin-2 receptor), cleavage of the receptor provides a mechanism for regulating the signal responsiveness of cells (37). In most cases the functional significance of cleavage of nonsignaling receptors is not known, but it could be a mechanism of rapidly down-regulating the receptor.

The extracellular region of the TfR proximal to the transmembrane domain (Cys-89 through Arg-120) appears to be relatively unstructured in that it is susceptible to several cellular and extracellular proteases. The soluble form of the TfR found in blood is cleaved at Arg-100 (43, 44) and is partially protected by O-linked glycosylation at Thr-104 (5, 6, 10). When cells are treated with low concentrations of trypsin the TfR is released by cleavage at Arg-120 (45). In this study, we have identified a third cleavage site at Gly-91 when membrane-proximal portion of the TfR cytoplasmic domain is deleted. Because the soluble TfR remains a dimer and is fully capable of binding transferrin, the region between Cys-89 and Arg-120 does not appear to play a critical role in the structure of the TfR.

In this set of studies we have demonstrated that alteration of the cytoplasmic domain of the TfR results in its enhanced cleavage. The cleavage occurs in the ectodomain of the molecule close to the membrane. Deletion of the portion of the TfR cytoplasmic domain proximal to the transmembrane region correlates with the cleavage of the extracellular domain. Pulse-chase and biotinylation studies were used to determine that unlike the cleavage of the TfR at the other protease susceptible sites, this cleavage occurs in the biosynthetic pathway. It correlates with the kinetics of the acquisition of endo H resistance, indicating that it occurs early in the biosynthetic pathway. The full-length TfR and the Δ3–29 TfR transit through the endoplasmic reticulum and Golgi compartments quickly and are not cleaved significantly. The extent of cleavage of the other TfR mutants, Δ3–29 TfR, Δ3–39 TfR, and Δ3–59 TfR, correlates with slowed exit from the endo H-sensitive compartments. Thus the most cleavage was seen in the mutant TfR lacking all but three amino acids on the cytoplasmic domain. Mutant TfRs lacking the membrane-proximal portion of the cytoplasmic domain were the slowest to exit the endo H-sensitive compartment and the most susceptible to the protease. Cleavage occurs between two hydrophobic amino acids consistent with the possible involvement of the ER signal peptidase. In agreement with this hypothesis is an observation by Hoe and Hunt (46) that a mutated form of the TfR, unable to exit the ER, was cleaved at Gly-91. A similar cleavage close to the transmembrane domain was seen with the H2α subunit of the asialoglycoprotein receptor when expressed in the absence of the subunit (42). In this case, the subunit was retained in the ER and cleaved.

Selective transport of proteins out of the ER was first proposed in the 1980s (47, 48). Different transport kinetics were observed between two retroviral membrane proteins (47). In hepatocytes, serum proteins varied widely in the acquisition of endo H resistance, ranging from α1-antitrypsin (t1/2 = 25 min) to transferrin (t1/2 = 180 min) (48). The time to transit through the Golgi was relatively uniform (~20 min). Evidence for concentration packaging of proteins into transport vesicles and potential transport receptors that facilitate protein exit was sparse. Contrary to this hypothesis, Wieland and colleagues (49) presented evidence that small peptides could transit through the ER quicker than newly synthesized proteins. These results were interpreted to mean that protein folding was the rate-limiting step. In the absence of retention, there was bulk flow from the ER to the Golgi. In keeping with this idea, proteins that fold at a slower rate are retained in the ER associated with resident ER chaperone proteins (50). Rose and Bergmann (51) demonstrated that deletions in portions of the cytoplasmic domain of vesicular stomatitis virus G (VSV-G) could slow the exit of this protein from the ER without influencing the folding kinetics. Balch and co-workers (52) have again proposed a specific concentrative step to be involved in the exit of VSV-G from the ER. This evidence is based on quantitation of the amount of VSV-G in vesicles budding from the ER by electron microscopy. Most recently Nishimura and Balch (53) demonstrated that a diacidic signal (Asp-X-Glu) was required for efficient exit of VSV-G from the ER. The transmembrane-proximal portion of the TfR (amino acids 29–59) which appears to participate in efficient transport of the TfR out of the endo H-sensitive compartment contains the sequence Asp-Glu-Glu-Glu (amino acids 43–48). Thus, both the folding kinetics and cytoplasmic domains of proteins can influence the rate of exit from the ER.

Future work is aimed at distinguishing between the possibilities that specific signals in the membrane-proximal portion of the cytoplasmic domain influence the concentration of the TfR into vesicles exiting the ER or alter the kinetics of the TfR luminal domain folding.

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The TfR Cytoplasmic Domain Affects Ectodomain Cleavage

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