Evidence for Distinct Saturable Components of Clathrin-mediated Endocytosis: Recognition of Different Tyrosine-based Internalization Signals

by

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CERTIFICATE OF APPROVAL

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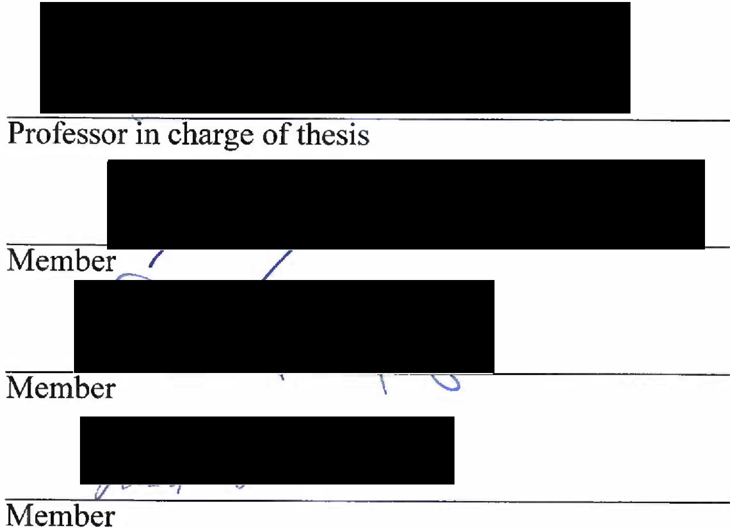

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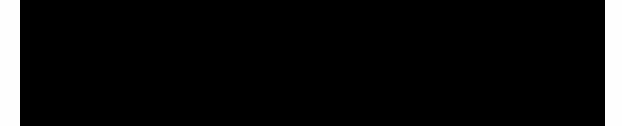

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ABSTRACT

Clathrin-mediated endocytosis, the process by which cells selectively internalize various nutrients, hormones and growth factors, has been the primary source of study for many labs in the past couple decades. The mechanisms behind the sequestration of plasma membrane receptors destined for internalization remain to be fully understood, and many of the players have yet to be discovered. Recognition of a specific internalization signal present in the cytoplasmic domain of receptors and proteins utilizing this pathway is an important first step for rapid and efficient endocytosis to occur. Though the research on the structure and function of the internalization signal is extensive, the details of receptor capture in clathrin-coated pits remain incomplete.

One of the approaches taken in this thesis is to perform a series of quantitative studies using live cells that are overexpressing a single wild-type form of a receptor, and measuring the competitive effects on other endogenous receptors undergoing endocytosis. Through the course of these studies three separate stable HeLa cell lines were generated that can be induced to overexpress either the TfR, the LDLR, or the EGFR in response to the exclusion of tetracycline from the media. When the TfR is overexpressed, a decrease in the rate of Tf endocytosis per surface TfR is measured when compared to uninduced cells. These data suggest that the decrease in the rate of TfR internalization is due to either the saturation of endocytosis, or a slowed recycling event. To eliminate the second possibility, the rate of exocytosis was measured in a simultaneous set of induced and uninduced TfR cells and demonstrated to remain unchanged. Using similar techniques with the other two cell lines, results were obtained that demonstrate that endocytosis of

each of the three tested receptors (TfR, LDLR, or EGFR) can saturate endocytosis for themselves when overexpressed.

After having established saturable endocytosis for each of the three receptors, the next step was to see if saturation of one receptor could affect the rate of endocytosis of other endogenous receptors. This time, a single receptor was overexpressed, and the rate of endocytosis or surface distribution of other endogenous receptors was measured. The finding was that none of the three receptors tested had any overt effect on the internalization of the others. The conclusion drawn from these results was that the TfR, LDLR and EGFR each require a component of the endocytic machinery that is present in limiting amounts and is distinct for each receptor.

Though the evidence presented herein suggests a role for at least three distinct recognition "devices", the question remains: are recognition components shared at all between receptors, or is there a different molecule necessary for the recognition of each and every protein utilizing clathrin-mediated endocytosis? By taking advantage of the phenomenon of the surface distribution shift that is seen in a system that is saturated for endocytosis, Lamp-1 was shown to compete with the TfR, but not with the LDLR. This lends support to the idea that there exists at least some amount of homogeneity in the recognition of tyrosine-based sorting signals.

In an attempt to understand the structure of the TfR internalization signal, the cytoplasmic domain has been expressed and purified from a bacterial expression system. Circular dichroism analysis reveals a flexible peptide lacking very little secondary structure except for nearly 40% β-turn. This supports earlier predictions that the YTRF amino acid sequence comprising the TfR internalization signal would reside in a tight turn.

Despite the evidence for internalization signal interactions with clathrin adaptor complexes, no association of the TfR cytoplasmic domain with purified clathrin coats is detected *in vitro*.

ABBREVIATIONS

Following is a list of abbreviations used in this thesis:

Tf	Transferrin
TfR	Transferrin Receptor
LDLR	Low Density Lipoprotein Receptor
ASGPR	Asialoglycoprotein Receptor
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
CI-M6PR	Calcium-independant Mannose-6-Phosphate Receptor
CD-M6PR	Calcium-dependant Mannose-6-Phosphate Receptor
InsR	Insulin Receptor
β-AR	β-Adrenergic Receptor
LAP	Lysosomal Acid Phosphatase
AP1	Clathrin Adaptor Protein-1
AP2	Clathrin Adaptor Protein-2
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
FITC	Fluorescein conjugate

INTRODUCTION

All living cells, divided from the outside world by a phospholipid bilayer, interact with and sample the environment through a number of complex cellular processes. In general, endocytosis is the process by which cells internalize soluble molecules and cell surface proteins, and can be subdivided into distinct types. Clathrin-mediated endocytosis, the focus of this introduction, is the primary mechanism utilized by cells to selectively internalize various nutrients, hormones, and growth factors. Other forms of endocytosis, including pinocytosis (fluid-phase uptake) and phagocytosis (ligand-induced internalization of large particles) will not be discussed.

The clathrin-mediated endocytosis pathway is used by a number of plasma membrane receptors to transport nutrients into the cell, or as a method of receptor downregulation. Many nutrient receptors are constituitively internalized, regardless of whether or not a ligand is bound, as is the case with the TfR, LDLR, and ASGPR (for review see (Goldstein et al., 1985)). These receptors spend a majority of the time that they are on the cell surface clustered in coated pits, targeted by a signal present in their cytoplasmic domain. After internalization, receptors dissociate from their ligand in the endocytic vesicle or early endosome as a result of a decreased pH. The abandoned ligand can then be utilized by the cell while the internalized receptor is recycled to the plasma membrane. In the case of the TfR, its ligand transferrin remains bound throughout the recycling process (Morgan, 1979). Iron is lost from transferrin at low pH in the early endosome and is transported from the lumen to the cytoplasm by an unknown

mechanism. Upon returning to the surface of the cell, the empty receptors can bind new ligand and begin another round of endocytosis.

Signalling receptors, like the EGFR and the InsR, do not undergo constituitive recycling. Instead these hormone receptors remain diffusely distributed on the plasma membrane until after ligand binding, at which point they rapidly cluster into coated pits (Carpentier et al., 1992; Willingham et al., 1983). Interaction with the clathrin coat requires multiple events to occur following ligand activation. For example, when EGF binds to EGFR, the tyrosine kinase in its cytoplasmic domain becomes active. It is thought that tyrosine phosphorylation of some component of the endocytic machinery, possibly the EGFR substrate Eps15, leads to the recruitment of this receptor to clathrin-coated pits (van Delft et al., 1997; Wiley et al., 1991). Likewise, InsR internalization requires activation of its tyrosine kinase domain, and subsequent phosphorylation of annexin II, a member of the calcium- and phospholipid-binding family of proteins (Biener et al., 1996). In both cases, the exact mechanism of recruitment and internalization is not well understood.

Clathrin Coats

Entry into clathrin-coated pits is the first step of receptor-mediated endocytosis, and is the point where molecules destined for internalization are separated from other non-endocytosing proteins. Clathrin coats, present on coated pits and coated vesicles, appear as an electron dense area surrounding the membrane on the cytoplasmic side, and under high resolution appears as a "lattice" structure. The composition of coats was first characterized by Barbara M. F. Pearse in 1975, when she purified coated vesicles from

pig brain cytosol, and analyzed the product by SDS-PAGE (Pearse, 1975). She described a predominant protein species of 180 kDa in size that she named clathrin. Clathrin is a fibrous protein that exists as a triskelion, made up by three heavy chains (180 kDa each) and three light chains (35-40 kDa each) (Figure 1a). When the triskelions associate with each other, they assemble into lattice-like polyhedral cages.

Several years later, it was discovered that other proteins co-purified with clathrin (Pearse, 1978). These proteins, termed clathrin adaptors, were shown to promote the assembly of clathrin coats *in vitro* (Keen et al., 1979; Zaremba and Keen, 1983), and were found to be necessary for the attachment of clathrin to the membrane (Unanue et al., 1981). Two of the most thoroughly studied adaptors, AP1 and AP2, are made up of four heteromeric subunits each (see Figure 1b and Table I below), and are known to promote the assembly of clathrin at either the Golgi complex or the plasma membrane, respectively (for reviews, see (Pearse, 1990; Robinson, 1994)). The large ~100 kDa subunits are typically described as consisting of three domains: the amino-terminal "brick"-like core (or head), the short protease-sensitive "hinge" region, and the carboxy-terminal ear.

Table I. *Summary of Adaptor Subunits*

AP1 subunits (TGN)	AP2 subunits (Plasma Membrane)	% Identity between AP1 and AP2 subunits	Molecular Weight
γ-adaptin	α-adaptin	25%	~100 kDa
β1-adaptin	β2-adaptin	85%	~100 kDa
AP47 or μ_1	AP50 or μ_2	40%	~50 kDa
AP19 or σ_1	AP17 or σ_2	45%	~20 kDa

Modified from (Robinson, 1994).

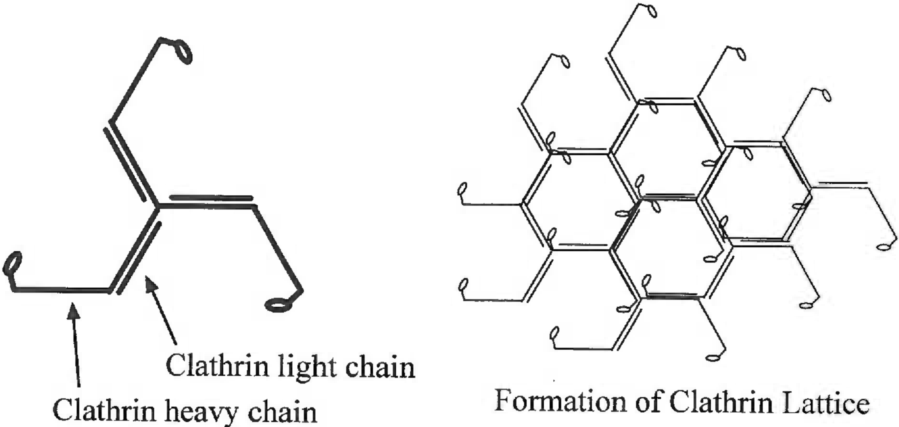


Figure 1a. *Clathrin Triskelion*

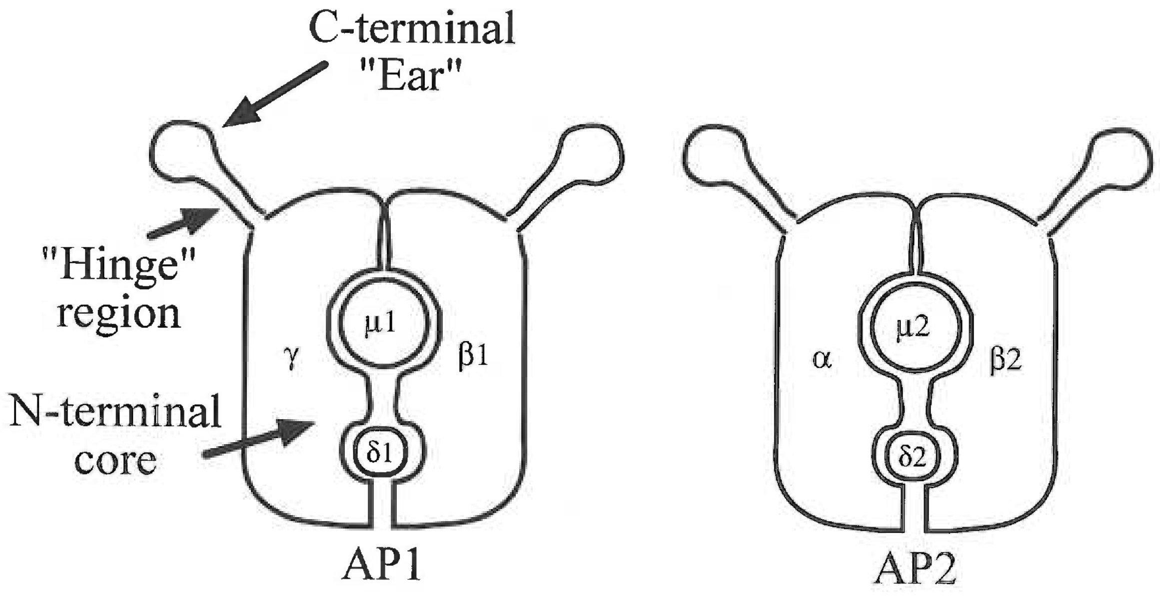


Figure 1b. *Subunits of AP1 and AP2*

Since β2 and β1 share the most homology, it was speculated that these subunits of AP2 and AP1 would contain the binding site for clathrin (Ahle et al., 1988). Indeed, these adaptor subunits, either purified from coated vesicles or recombinantly expressed in *Escherichia coli*, were able to bind to clathrin triskelia or preformed clathrin cages *in vitro* (Ahle and Ungewickell, 1989; Gallusser and Kirchhausen, 1993; Schroder and Ungewickell, 1991). More recently it was shown that the physical clathrin binding site, at least for β2-adaptin, resides in the flexible hinge region (Shih et al., 1995).

Both AP1 and AP2 are able to bind clathrin, but are somehow targeted to different membranes. Early experiments to try and locate the membrane targeting signal for these adaptors began when researchers took advantage of the protease-sensitive hinge region to remove the "ears" from AP2 by trypsin or elastase treatment. The "earless" AP2 complex, which was still able to self-associate *in vitro* (Beck and Keen, 1991), suffered no loss in ability to associate with the plasma membrane (Peeler et al., 1993). In other work, Robinson and coworkers showed that the ear domains could be swapped between α- and γ-adaptin, without affecting the targeting of the adaptor complex (Robinson, 1993). All of this suggested that the membrane targeting signal had to reside either in the core domain of the adaptins, or in the smaller subunits of the complex.

To further characterize the specific location of the membrane targeting signal, α- and β2-adaptins were purified from the smaller μ_2 and δ_2 subunits and tested for their ability to bind clathrin-stripped membranes (Chang et al., 1993). Only α-adaptin, as determined by radioimmunoassay, was competent to bind the plasma membrane. Further studies using multiple chimeric constructs between α- and γ-adaptin, revealed a stretch of 200 amino acids, proximal to the amino terminal end, that was necessary for efficient

binding to the appropriate target membrane (Page and Robinson, 1995). Though the importance of this region for membrane binding is clear, it does not necessarily rule out the possibility for multiple binding sites, or specific contributions from the other subunits.

The plasma membrane binding site for AP2 has yet to be fully understood, but probably constitutes a combination of different structures. The importance of an integral membrane protein became apparent when Mahaffey and colleagues were able to show that elastase digestion of the cytoplasmic face of an intact membrane effectively removed a high affinity AP2 binding site (Mahaffey et al., 1990). Previous to this, it had been hypothesized that receptor tails containing internalization signals provided the necessary site for recruitment of AP2 to the plasma membrane (Pearse and Bretscher, 1981). Two independant groups were able to show that receptor tails taken from either the LDLR or CI-M6PR and attached to beads on a affinity matrix column, could weakly bind AP2, but only when receptor sequences necessary for internalization were left intact (Glickman et al., 1989; Pearse, 1988). Similarly, the LAP cytoplasmic domain could bind AP2 on a column (Sosa et al., 1993), and the ASGPR specifically bound to β2-adaptin in an *in vitro* blotting assay (Beltzer, 1991). In other work, peptides representing the cytoplasmic domain sequence of the predominant subunit of the ASGPR could inhibit adaptor membrane binding, if adaptors were first preincubated in micromolar amounts of peptide (Chang et al., 1993). The blocking ability of the peptide was destroyed when the single tyrosine was mutated to an alanine. Despite the specificity of all of these associations, the micromolar binding affinities were too low to account for nanomolar plasma membrane binding of AP2. Further, attempts at using high densities of receptor

cytoplasmic domains to force the increased recruitment of AP2 to the plasma membrane have proven unsuccessful (Santini and Keen, 1996; Warren et al., 1997). The proposal that there exists an AP2 docking site separate from receptor cytoplasmic domains remains intact, but identification of a candidate protein has yet to be determined.

Other proteins have been implicated as adaptors in clathrin-mediated endocytosis. For example, the protein β-arrestin has been shown to be important for the internalization of the β-adrenergic receptor (Goodman et al., 1996). The β-arrestin protein appears to act by circumventing the function of AP2 by interacting directly with the cytoplasmic domain of β-AR and clathrin (Goodman et al., 1997; Goodman et al., 1996; Krupnick et al., 1997). In another example, the Eps15 protein, a phosporylation substrate of the EGFR, has been suggested to be involved in the endocytosis of this receptor (Benmerah et al., 1996; van Delft et al., 1997).

Besides adaptors and clathrin, other proteins have been identified that participate in the proper functioning of the coated pit. First identified as a microtubule-bundling protein (Shpetner and Vallee, 1989), dynamin was shown to have 69% identical amino acid sequence to the *shibire* gene product, an endocytosis-defective mutation in *Drosophila* (van der Bliek and Meyerowitz, 1991). When GTPase-defective mutants of dynamin were generated and stably transfected into HeLa cells (Damke et al., 1995), large accumulations of invaginated coated pits were observed to collect on the plasma membrane (Damke et al., 1994). Damke and coworkers also noted that cytosolic AP2, and not AP1, was depleted in these cells. This suggested that dynamin was somehow involved in the mechanism of invagination of coated pits at the plasma membrane. Taken together with evidence of dynamin self-assembly into rings *in vitro* (Hinshaw and Schmid,

1995), a role was suggested for dynamin in the constriction and pinching off of clathrin-coated pits.

The elucidation of all the components of the clathrin-coated pit is far from complete, and specific details of pit formation and the physical process of invagination and internalization continue to be compiled. Ultimately though, the mechanism behind recognition and recruitment of receptors for endocytosis depends heavily on short sequences of amino acids in the cytoplasmic domains of these proteins, termed internalization signals.

Internalization Signals

The earliest indication of a signal for endocytosis came when Brown and Goldstein described mutations of the LDLR in Familial Hypercholesterolemia (FH) (Goldstein et al., 1985). Though many of the mutations seen in FH patients affected the processing of LDLR or its ability to bind the ligand LDL, a few were described that prevented the LDLR from clustering into coated pits. Analysis of each of these "internalization-defective" mutants revealed modifications in the cytoplasmic domain (Goldstein et al., 1985; Lehrman et al., 1985). Though two of these were deletions, the mutant that proved to be the most interesting was a $Tyr^{807} \rightarrow Cys$ substitution (Davis et al., 1986). Referred to as the JD mutation, this single amino acid change had a drastic effect on the ability of the LDLR to cluster into coated pits, displaying a rate of internalization that was at least 4-fold less than that seen with the wild type receptor.

The loss of high efficiency endocytosis of the LDLR could not immediately be attributed to the absence of a tyrosine, or the presence of a new cysteine. The

importance of tyrosine in the internalization signal motif became more clear when Lazarovits and colleagues demonstrated that hemaggluttinin, an influenza virus transmembrane protein which is normally excluded from coated pits, could be targeted for endocytosis by insertion of a tyrosine residue in the cytoplasmic domain (Lazarovits and Roth, 1988). The tyrosine insertion was only effective in one of the three positions tested, suggesting that the sequence context is important, and that other amino acids may play a role in the formation of a functional internalization signal. When the cytoplasmic domain of the LDLR was subjected to mutational analysis, the amino acids Asn^{804} and Pro^{805} were also determined to be important for the correct targeting of the receptor for internalization (Chen et al., 1990). They demonstrated that the sequence NPXY (where X represent any amino acid) was conserved among LDLRs from six different species, and proposed that the amino acid tetramer represented an internalization signal motif.

Characterization of the endocytic signal for the TfR began when Rothenberger and colleagues demonstrated that a cytoplasmic deletion of 36 amino acids led to a 2- to 5-fold decrease in the rate of endocytosis of ^{125}I-labeled transferrin in mouse L-cells (Rothenberger et al., 1987). Further localization of the signal was achieved by successive smaller deletions, amino acid substitutions, and alanine scanning techniques (Jing et al., 1990; McGraw and Maxfield, 1990). Amino acids 20-23, containing the sequence YTRF, were determined to constitute the necessary signal for endocytosis of the TfR. Interestingly, insertion of a tyrosine at a second site within the cytoplasmic domain could actually rescue an internalization-defective Tyr^{20}-Cys mutation (McGraw et al., 1991).

To date, the internalization signals for a large number of membrane proteins have been characterized, including CI-M6PR (Lobel et al., 1989), CD-M6PR (Johnson et al.,

1990), IgG-R (Miettinen et al., 1989), polymeric Ig-R (Mostov et al., 1986), ASGPR (Fuhrer et al., 1991; Geffen et al., 1993), β-amyloid precursor protein (Kang et al., 1987), Lamp-1 (Williams and Fukuda, 1990), Lamp-2 (Granger et al., 1990), β_2-adrenergic receptor (Valiquette et al., 1990), and Lysosomal Acid Phosphatase (Lehmann et al., 1992). Each one of these signals are 4 to 6 amino acids in length, and consist of either the "LDLR-like" NXXY motif, or the "TfR-like" YXX∅ motif (where X is any amino acid, and ∅ represents any hydrophobic residue).

Though there is some heterogeneity among internalization signals from different proteins, one common theme is that they contain a tyrosine that has been shown to be crucial for the proper clustering of receptors into coated pits. Another common theme may lie with the formation of secondary structure by the cytoplasmic domain in an aqueous environment. In an attempt to understand the structure of the internalization signal, the TfR sequence, YXRF, and LDLR sequence, NPXY, were compared against a database of protein crystallographic structures at the Brookhaven Protein Data Bank, using a non-biased search pattern (Collawn et al., 1990). Interestingly, a majority of the analogs to the two sequences favored secondary structure conforming to a tight turn. In other work, independent groups generated synthetic peptides from the cytoplasmic domains of LDLR, LAP, and InsR, and analyzed them by NMR (Backer et al., 1992; Bansal and Gierasch, 1991; Eberle et al., 1991). Each of these groups found that their peptides formed a structure that correlated with a type I β-turn, supporting earlier predictions. These studies provided the first physical evidence of a common structure, and brought forth the idea that recognition by the endocytic machinery may in fact be structure specific.

Tyrosine-based internalization signals have been studied in great detail, and appear to provide the targeting information necessary for coated pit clustering and internalization of many different types of receptors and proteins. However, the tyrosine-based motif is unlikely to be the only type of endocytosis targeting information. Another internalization signal, which has come into evidence more recently, is the dileucine motif. This signal, made up of either two consecutive leucine residues, or an isoleucine-leucine residue pair, was originally identified as a lysosomal targeting signal from CD3-δ or CD3-γ that could promote rapid and efficient internalization when attached to the cytoplasmic domain of Tac antigen (interleukin-2 α-chain) (Letourneur and Klausner, 1992). The dileucine motif has since been implicated in the endocytic targeting of various other proteins, including CD4 (Foti et al., 1997; Shin et al., 1991), InsR (Haft et al., 1994), β_2-AR (Gabilondo et al., 1997), and the GLUT4 glucose transporter (Corvera et al., 1994; Verhey and Birnbaum, 1994). Unlike the tight-turn structure adopted by the tyrosine-based motifs, the dileucine motif is thought to exist in an extended structure, closely resembling an α-helix (Sandoval et al., 1994).

Receptors destined for clathrin-mediated endocytosis, must contain within their cytoplasmic domain, some combination of tyrosine- and/or dileucine-based targeting information. This suggests that the amino acids which make up the internalization signal might interact with components of the endocytic machinery in a specific way to trap receptors in clathrin-coated pits. The next section will deal with what is known about these interactions, and describe some of the predominant hypotheses regarding receptor sequestration.

Recognition of the Internalization Signal

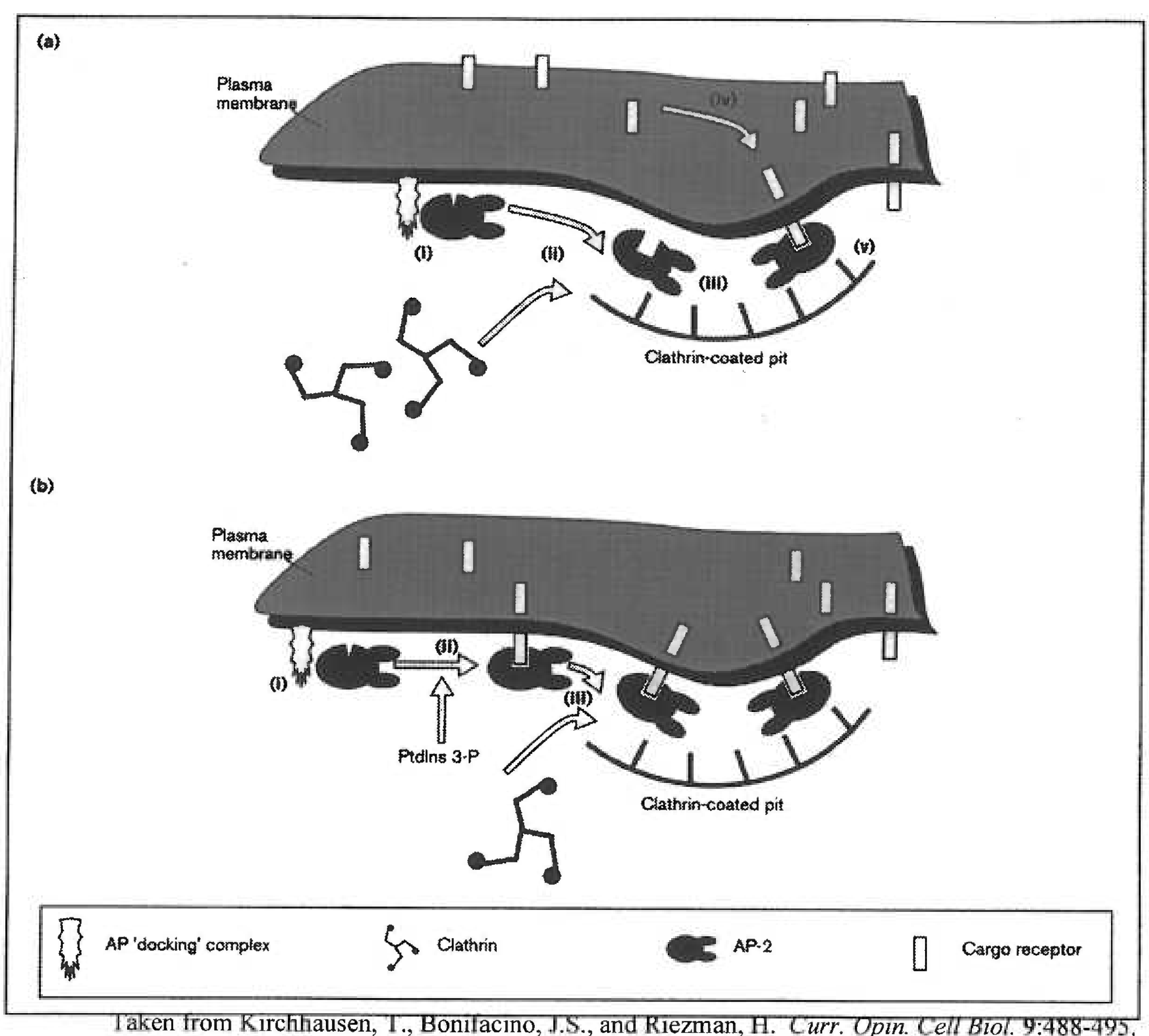
In an attempt to discern the molecule or molecules that interact directly with the internalization signal, Ohno and colleagues performed a two-hybrid screen of a mouse cDNA library (Ohno et al., 1995). As "bait" they used a triple repeat of the tyrosine-based motif from TGN38 (SDYQRL). The resulting two clones that were isolated from the library both matched the sequence of the μ_2 subunit (medium chain) from AP2. Interaction of the two clones with the bait was fully dependant on the presence of tyrosine and leucine in the sequence, confirming the specificity of the interaction. Sequence from both μ_2 and μ_1 was shown to interact with triple repeats of tyrosine-based motifs from several different transmembrane proteins, but not with the YTRF internalization signal from TfR, or the DKQTLL dileucine motif from CD3-γ. Interaction of the TfR internalization signal with μ_2 however, could be detected if the YTRF sequence was transposed onto the end of the TGN38 cytoplasmic domain.

Studies utilizing photoreactive peptides containing either the SDYQRL sequence, or the internalization defective SDAQRL sequence, have further defined the specificity of the μ_2 interaction to the presence of the tyrosine-based signal (Rapoport et al., 1997). Additionally, an increase in binding affinity between peptide and μ_2 was seen when AP2 contained in clathrin coats was used instead of free AP2, or when phosphoinositides were included in the binding reaction. However, the ability of the internalization signal to bind μ_2 is strongly dependant on both its position and the context of amino acids surrounding it (Boll et al., 1996; Ohno et al., 1996).

Other evidence for direct binding between adaptor proteins and sorting signals, comes from *in vitro* binding assays from two labs that utilized surface plasmon resonance

(SPR) technology (Heilker et al., 1996; Honing et al., 1996). SPR is a spectroscopic technique that allows the measurement of minute changes in protein-protein interactions on a thin metal film (Jonsson et al., 1991). Both groups demonstrated measurable binding of AP1 and AP2 to tyrosine-based sorting signal containing peptides that were immobilized on a BIAcore sensor chip. Binding was dependant on intact internalization signals, showing a moderate decrease in affinity when peptides lacking the crucial tyrosine were used. Interestingly, the presence of two leucines tagged to the end of a peptide lead to an increase in binding, even over that seen with the tyrosine-based signals (Heilker et al., 1996). Overall, the conclusion drawn was that the interaction seen between receptor tails and adaptor proteins of the clathrin-coat reflected the mechanism for receptor sequestration *in vivo*.

A popular model for the clustering of receptors into clathrin-coated pits is shown in figure 2, taken from a recent review by Kirchhausen et al. (Kirchhausen et al., 1997). This model predicts that AP2 is recruited to the plasma membrane through high affinity binding to an as yet unidentified AP2 docking complex. Once at the membrane, low affinity associations with receptor cytoplasmic domains can occur, either before or after AP2 recruits clathrin to the clathrin-coated pit.



Taken from Kirchhausen, T., Bonifacino, J.S., and Riezman, H. *Curr. Opin. Cell Biol.* 9:488-495.

Figure 2. *Model of Clathrin-mediated Endocytosis*

To answer some of the questions behind the mechanism of internalization signal recognition, the problem can be approached from the perspective of saturable binding. That is, if a component of the endocytic machinery responsible for binding/recognition of the endocytic signal can be saturated with an excess of signal, then we should be able to identify other molecules that require this component, by measuring a reduction in the rate of endocytosis of these molecules. A similar idea was used by H.S. Wiley to identify a saturable pathway for the endocytosis of the EGFR (Wiley, 1988). He demonstrated that EGFR internalization became saturated in A431 cells when an excess of the ligand EGF was added to the medium. This treatment caused an effective increase of the total number of EGFRs clustering into coated pits, and led to an overall decrease in the rate of internalization of EGF per activated surface receptor.

The purpose of this thesis is threefold. First, I want to demonstrate that the endocytic mechanism for the internalization of constituitively recycling receptors is a saturable process. This would suggest that some limiting component or components of the endocytic machinery are necessary for rapid and efficient endocytosis. Second, I will show that are at least three different pathways for the recognition and internalization of receptors bearing tyrosine-based motifs. Finally, I will examine the structure and function of the TfR cytoplasmic domain, as it applies to the internalization signal.

In chapter 1, the system for overexpression of the TfR in a human cell line is developed, and its effects on endocytic saturation, and competition with the EGFR are examined. AP2 and clathrin recruitment are also discussed. This paper was published in the *Journal of Biological Chemistry* (1997) 272(4):2116-2121.

In chapter 2, the generation of two new cell lines capable of overexpressing either the LDLR or EGFR, allow a thorough examination of the characteristics of individual receptor saturation, and competition between different receptors. This paper has been submitted for publication.

The last chapter will focus on the structure and function of the TfR cytoplasmic domain, and embodies the text of a manuscript in preparation.

CHAPTER 1

Saturation of the Endocytic Pathway for the Transferrin Receptor Does Not Affect the Endocytosis of the Epidermal Growth Factor Receptor

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SUMMARY

Cell surface receptors that undergo clathrin-mediated endocytosis contain short amino acid sequences in their cytoplasmic domain that serve as internalization signals. Interactions between these sequences and components of the endocytic machinery should become limiting upon overexpression of the constitutively recycling transferrin receptor (TfR). A tetracycline responsive system was used to induce overexpression of the TfR up to 20 fold in HeLa cells. Internalization assays indicate the rate of ^{125}I-Tf uptake per surface TfR is reduced by a factor of four in induced cells. Consistent with endocytosis being the rate limiting step, the TfRs shift from an endosomal to more of a plasma membrane distribution with TfR overexpression. The clathrin-associated protein, AP2, has been proposed to interact directly with the cytoplasmic domain of many receptors, yet no changes in the amount or distribution of AP2 were detected in induced cells. The internalization rate for epidermal growth factor receptor was also measured, with or without induction of TfR expression. Even though endocytosis of the TfR is saturated in induced cells, ^{125}I-labeled epidermal growth factor continues to be internalized at a rate identical to that seen in uninduced cells. We propose that there are different limiting steps for the endocytosis of these two receptors.

INTRODUCTION

Receptor-mediated endocytosis is a mechanism by which a number of cell surface receptors and their ligands are internalized by the cell. The process involves the concentration of specific plasma membrane proteins into clathrin-coated pits, followed by rapid internalization and delivery to early endosomes. The exact mechanism by which sequestration of surface receptors occurs is unclear, though it appears that short amino acid sequences in the cytoplasmic domains of captured proteins act as internalization signals that are necessary, and in some cases sufficient for endocytosis (Chen et al., 1990; Collawn et al., 1991; Collawn et al., 1993; Collawn et al., 1990; Davis et al., 1986; Davis et al., 1987; Lazarovits and Roth, 1988).

Internalization signals have been identified for a large number of proteins which span several different classes of receptors (reviewed in (Trowbridge et al., 1993)). A common tyrosine-based motif, which has been proposed to form secondary structure comprising a beta turn, provides a potential functional link between these signals. The structurally similar signals are believed to interact directly with proteins of the coated pit, presenting the possibility of competition for internalization among receptors. The clathrin-associated protein-2 (AP2) has been proposed to be the primary candidate for interacting with the cytoplasmic domains of receptors targeted for internalization (Chang et al., 1991; Pearse, 1985; Pearse, 1988).

We are investigating the mechanisms by which receptors are recruited to clathrin-coated pits. Using the tetracycline-responsive promoter cell expression system developed by Gossen and Bujard (Gossen and Bujard, 1992), we are able to show that by

overexpressing an epitope-tagged TfR in HeLa cells, the endocytic mechanism can be saturated such that the rate of internalization for transferrin (Tf) per surface TfR decreases at high TfR concentrations. The expression and distribution of AP2 remains unaltered in induced cells. Additionally, we can demonstrate that although the pathway of endocytosis for TfR is saturated, the activated EGFR continues to be internalized at a rate similar to uninduced cells. We conclude that there are different limiting steps for the endocytosis of these two receptors.

MATERIALS AND METHODS

Plasmids—The plasmid pCD-TR1, containing the sequence for the human TfR, was a gift from Dr. A. McClelland, Yale University (McClelland et al., 1984). The pUHD10-3 plasmid was a gift from Dr. M. Gossen and Dr. H. Bujard, Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH) (Gossen and Bujard, 1992).

Subcloning and Site-directed Mutagenesis—The human TfR cDNA coding region in pCD-TR1 is contained within a 2.5 kb BamHI-XbaI fragment. A HindIII site 900 bases from the start codon was used to generate the 1.5 kb HindIII-XbaI fragment, which was subsequently cloned into the M13mp18 vector for mutagenesis. Amersham's oligonucleotide-directed mutagenesis system was used to insert the sequence for the flag epitope (-DYKDDDDK-) (Hopp et al., 1988) immediately prior to the termination codon. The modified C-terminal portion of the human TfR was excised with HindIII-BamHI, and ligated with the BamHI-HindIII N-terminal fragment into the pUHD10-3 BamHI site, resulting in the fTfR/pUHD10-3 construct.

Iodination—Either 1600 μg of human holo-Tf (Intergen) or 5 μg of human EGF (Bethesda Research Laboratories) was used in a 50 μl reaction of 225 mM sodium phosphate buffer (pH 7.0), 0.5 units of lactoperoxidase (Sigma), 2 mCi carrier-free $Na^{125}I$ (New England Nuclear), and 0.003% H_2O_2 (Sigma). The mixture was allowed to react for 5 minutes at room temperature before adding 50 μl of 2% blue dextran in phosphate buffered saline

(PBS). The protein was separated from unreacted $Na^{125}I$ on a 2 ml desalting column (Pierce).

Immunodetection—Cells grown on coverslips were washed several times with PBS, fixed for 15 minutes at room temperature with 3% paraformaldehyde, and washed an additional two times with PBS (Bacallao and Stelzer, 1989; McDowell and Trump, 1976). Cells on coverslips were incubated for 1 hour at room temperature with a 1:50 dilution of sheep anti-TfR (characteristics similar to goat anti-TfR described earlier (Enns et al., 1983)) followed by a second 1 hour incubation at room temperature with a 1:50 dilution of FITC-labeled swine anti-goat secondary antibody (TAGO Inc.). Reducing SDS-polyacrylamide gel electrophoresis and Westerns were as previously described (Williams and Enns, 1991). An anti-peptide antibody (#1868) to the eight amino acid flag epitope sequence, coupled to keyhole limpet hemocyanin was generated in rabbits and affinity purified (Immunodynamics, La Jolla CA). Molecular weight markers (Sigma) included myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa).

Cell Culture—The fTfR20-2 cell line was generated by introducing the fTfR/pUHD10-3 plasmid into tTA HeLa cells (provided by Dr. Sandra L. Schmid, Scripps Research Institute) by calcium phosphate precipitation (Chen and Okayama, 1987). Selection was achieved by co-transfecting with pBSpac (de la Luna et al., 1988) which contains the resistance gene for the antibiotic puromycin. All colonies were recloned to insure a pure

cell line. Selected colonies were screened by gel electrophoresis and Western blot analysis with #1868 rabbit anti-flag and sheep anti-TfR primary antibodies.

Transfected cells (fTfR20-2 cells) were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 400 μg/ml G418 (Geneticin), 400 ng/ml puromycin and 2 μg/ml tetracycline. To induce overexpression of the TfR, tetracycline was excluded from the medium, and cells were allowed to grow for at least 96 hours (unless otherwise indicated).

Uptake Protocol—The rate of ^{125}I-Tf uptake was measured using a method modified from McGraw and Maxfield (McGraw and Maxfield, 1990). fTfR20-2 cells grown to confluency in 6-well plates (1×10^6 - 2×10^6 per well) were washed twice with wash medium (DMEM buffered with 20 mM HEPES, pH 7.4) and incubated for 15 minutes at 37°C. At time zero, 1 ml of either medium S (wash medium + 210 nM ^{125}I-Tf) or Medium NS (Medium S + 1 mg/ml unlabeled Tf) were added to 4 wells or 2 wells of a six-well plate, respectively. Each plate was incubated at 37°C and 5% CO_2 for 2, 4, 6, or 8 minutes, after which it was placed onto ice, and incubated with an acidic buffer (0.2 N acetic acid, 0.2 M NaCl) for 2 minutes at 4°C to remove any surface bound ^{125}I-Tf. Each well was washed four times with 4°C final wash (150 mM NaCl, 20 mM HEPES pH 7.4, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$), solubilized in 0.1 N NaOH, 0.1% Triton X-100 and counted on a gamma counter. The counts represent only the ^{125}I-Tf that was internalized during the 37°C incubation. The number of surface TfRs for each uptake experiment was determined by incubating 4 and 2 wells of a six-well plate in medium S and medium NS, respectively, on ice for 90 minutes. These wells are washed four times

with 4°C final wash, and solubilized as described. All results are corrected for specific measurements, by subtracting nonspecific uptake and binding amounts. TfR distribution was determined following a protocol described by McGraw and Maxfield (McGraw and Maxfield, 1990), except that ^{125}I-Tf was used at a higher concentration of 210 nM.

AP2 distribution—fTR20-2 cells were grown to confluency on 6-well plates pretreated with poly-D-lysine (Sigma). To expose the cytoplasmic face of the membrane, cells were freeze-fractured by placing plates onto ice for 15 minutes, washing once with 1 ml H_2O at 4°C, and placing at -80°C with 1 ml H_2O for approximately one hour. While thawing in a 37°C bath, each well was brought to a final concentration of Buffer E: 36.4 mM HEPES, 68.2 mM KCl, 4.1 mM Mg-acetate, pH 7.2, 1 mM dithiothreitol (DTT), 10 μM leupeptin (Sigma), 1 mM orthophenanthroline (Sigma), 0.5 mM benzamidine (Sigma), 2 μg/ml soybean trypsin inhibitor (Sigma) (Chang et al., 1993) or Buffer F: 0.5 M Tris-Cl, pH 7.0, 10 μM leupeptin, 1 mM orthophenanthroline, 0.5 mM benzamidine, 2 μg/ml soybean trypsin inhibitor, 1 mM DTT (Mahaffey et al., 1990). Attached membranes were scraped from the wells and the pooled samples were centrifuged at 1,600 x g for 10 minutes at 4°C followed by centrifugation of the supernatant at 90,000 x g for 45 minutes at 4°C. Membrane pellets (P) and whole cell lysates were solubilized in NET buffer (150 mM NaCl, 5 mM EDTA, pH 7.4, 10 mM Tris-Cl) + 1% Triton X-100. Samples representing equal numbers of cells were run on an SDS-polyacrylamide (8%) gel and transferred to nitrocellulose. Western blots were probed with either a mouse monoclonal antibody to AP2 (Clone 100/2, Sigma) or sheep anti-TfR, followed by

horseradish peroxidase-conjugated swine anti-mouse and swine anti-goat (Boehringer Mannheim), respectively.

RESULTS

Identification of two clones that express epitope tagged TfR under tetracycline-regulation. A cell line capable of overexpressing the TfR was needed in order to study the effect of TfR density on endocytosis. The tetracycline-responsive promoter system developed by Gossen and Bujard (Gossen and Bujard, 1992) was chosen because the expression of the TfR could be tightly controlled. The system involves the use of a stable HeLa cell line that expresses a fusion protein consisting of the tetracycline responsive element (tetR) from *E. coli*, and the VP16 activation domain from Herpes Simplex Virus (HSV). In the absence of tetracycline, the tetracycline transactivator (tTA) is free to bind tetracycline-operator sequences found in the promoter region of the pUHD10-3 plasmid, and promote transcription. When tetracycline is added to the cell media, the tTA fusion protein is blocked from interacting with the promoter, and transcription does not occur.

The human TfR sequence containing a flag-epitope tag at its carboxy-terminus (C-flag TfR) was subcloned into the pUHD10-3 vector. The fTfR/pUHD10-3 construct along with a puromycin selection vector (pBSpac (de la Luna et al., 1988)) were cotransfected into tTA expressing HeLa cells. Approximately 30 colonies were isolated, and screened by Western analysis for inducible expression of the C-flag TfR in the absence of tetracycline in the media. Induction of the C-flag TfR (94 kDa) along with a minor amount of unreduced TfR (180 kDa) was detected in two different clones using the anti-flag antibody (Figure 1, right panel). Since the promoter region by itself is silent in mammalian cells, no detectable expression of the C-flag TfR is seen at day 0. Anti-TfR shows endogenous levels of wild type TfR at day 0 of induction, and a distinct increase

of total TfRs (wild type and C-flag TfR) after 3 to 4 days (Figure 1, left panel). Clone fTfR20-2 was used in subsequent experiments.

Before quantitative ^{125}I-Tf uptake measurements of the fTfR20-2 clone as a population could be made, we wanted to show that expression of the C-flag TfR was homogeneously induced in all of the cells. fTfR20-2 cells were grown either in the absence or the presence of tetracycline, to represent induced, or uninduced cells, respectively. Surface TfR expression was visualized by incubating non-permeabilized fixed cells with an FITC-conjugated polyclonal antibody to the TfR. A more intense surface fluorescence labeling is apparent in induced cells (Figure 2, –Tet), indicating a greater number of TfRs. This level of fluorescence was seen uniformly in all cells for every microscopic field observed. Uninduced cells (Figure 2, +Tet) display a labeling pattern identical to the parent tTA HeLa cell line (data not shown), indicating full repression of the C-flag TfR protein.

Stable fTfR20-2 cells can be induced to express C-flag TfR at levels 10- to 20-fold over endogenous TfR. The binding of Tf was examined to quantitate the actual number of TfRs being expressed on the cell surface of the fTfR20-2 clone. Scatchard analysis was performed on uninduced and induced fTfR20-2 cells (Figure 3). Uninduced cells show binding sites equivalent to approximately 1.5 to 2.0×10^5 TfR per cell surface, and a KDa of Tf for the TfR of 3.1 nM. Cell surface binding sites on the order of 2.0×10^6 to 4.0×10^6 TfRs/ cell, a factor of 10-20 increase over endogenous TfR, are measured in induced cells. The KDa of Tf for the TfR measured in induced cells is lower (25.8 nM), suggesting that addition of the flag epitope to the C-terminus affects Tf binding to a

limited extent. All uptake assays were done at 210 nM ^{125}I-Tf to insure that the uptake assays reflected the rate of uptake, and not the rate of Tf binding. Bindings performed with either 210 nM or 420 nM ^{125}I-Tf yield similar results indicating that Tf binding was saturating and not the limiting factor in the uptake experiments (data not shown).

The rate of Tf uptake per surface TfR is decreased in induced fTfR20-2 cells. Rates of ^{125}I-Tf uptake were measured to demonstrate that the induction of TfR expression in the fTfR20-2 cell line is sufficient to saturate endocytosis. Uninduced or induced cells were incubated with a saturating amount of ^{125}I-Tf (210 nM) for 2, 4, 6, or 8 minutes at 37°C and 5% CO_2. Uninduced fTfR20-2 cells expressing 2 x 10^5 TfRs/ cell surface, display an endocytic rate of 0.39 Tf/ surface TfR/ min (Figure 4). In induced cells (2 x 10^6 TfRs/ cell surface), the measured rate was 0.10 Tf/ surface TfR/min, a significant 3.9 fold decrease. Control uptakes were performed on the parent tTA HeLa cell line, grown with or without tetracycline, to demonstrate that the effects we have measured were specific to the number of TfRs expressed on the surface. No significant difference in the rate of ^{125}I-Tf endocytosis was detected (data not shown).

The number of TfRs expressed on the surface was compared to the rate of ^{125}I-Tf uptake per surface TfR in order to determine whether endocytosis of the TfR could be saturated. We needed to vary the length of time that fTfR20-2 cells spend in the absence of tetracycline to control the number of TfRs but it was difficult to completely remove all of the tetracycline from plated cells by washing, resulting in sporadic induction. In contrast, turning off expression by plating induced cells onto 6-well plates, and adding tetracycline for varying periods of time, gave more precise control over TfR number.

The rate of EGF uptake per EGFR is unchanged between uninduced and induced cells. We wanted to determine how overexpression of the constitutively recycling TfR would affect the endocytosis of a triggered receptor, such as the epidermal growth factor receptor (EGFR). The EGFR has been shown to co-localize with the TfR in coated pits after binding its ligand, EGF (Hanover et al., 1985; Hopkins et al., 1985), making it a potential competitor with the TfR for common endocytic components. Using the same protocol as described for TfR uptake, we examined the rate of internalization of ^{125}I-EGF in induced and uninduced fTfR20-2 cells (Figure 7). Although induced cells are expressing nearly two million TfRs on the cell surface, a density that we have shown to impede the endocytosis of the TfR, the rate of endocytosis for EGFR is not significantly changed from that of uninduced cells (compare 0.17 to 0.16 EGF/ surface EGFR/ min). This result suggests that they do not share a common rate limiting factor(s) or pathway.

The distribution of the clathrin adaptor protein, AP2, remains unchanged in cells overexpressing TfR. Tyrosine-based motifs in the cytoplasmic domains of a number of receptors are involved in the concentration of these receptors into coated vesicles (Alvarez et al., 1990; Chen et al., 1990; Collawn et al., 1990; Davis et al., 1986; Iacopetta et al., 1988). AP2, a major component of the clathrin-coated pit, has been shown to interact specifically with a number of receptors and peptides targeted for internalization (Bansal and Gierasch, 1991; Chang et al., 1991; Eberle et al., 1991; Glickman et al., 1989; Nesterov et al., 1995; Sorkin et al., 1996; Sorkin et al., 1995; Sosa et al., 1993). Both Iacopetta and coworkers and Miller and coworkers have noted that either more clathrin-coated pits, or more clathrin lattices are associated with the plasma membrane of cells

expressing high numbers of TfR than in corresponding cells with fewer TfRs (Iacopetta et al., 1988; Miller et al., 1991) using electron microscopy to quantitate their results. If more clathrate structures are associated with the plasma membrane, then more AP2 should be associated too.

We were interested to see whether a large number of TfRs on the cell surface would lead to a redistribution of AP2 to the plasma membrane, as might be expected if AP2 is interacting with the cytoplasmic domain of the TfR. The amount of AP2 either attached to the membrane or present in the cytosol was measured in both uninduced and induced cells to determine the AP2 distribution (Figure 8). Using a protocol modified from Mahaffey and coworkers (Mahaffey et al., 1990), cells were freeze-fractured to expose the cytoplasmic face, and thawed in either a control buffer (buffer E), which preserves AP2 and clathrin binding to membranes, or a Tris buffer (buffer F) to dissociate clathrin and adaptor proteins from the membrane and show the total amount of AP2 in the cells. Cells were fractionated into pellet (P) containing membranes, and supernatant (S) containing cytosol by scraping and centrifugation. Samples representing an equal number of cells were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunodetected with an antibody to AP2 or to TfR. The levels of AP2 seen in the membrane (P) fractions of uninduced or induced fTfR20-2 cells washed with buffer E are similar. No AP2 was detected in membranes washed with buffer F indicating that the AP2 could be removed from the membranes as previously described (Mahaffey et al., 1990). A direct comparison of whole cell lysates demonstrated that the overall levels of AP2 expression remains unchanged, regardless of the number of TfRs. We conclude that AP2 is not actively recruited to the plasma membrane with increased

TfR expression and other factors control or limit the association of AP2 with the plasma membrane.

DISCUSSION

The mechanism by which specific plasma membrane proteins are selected for endocytosis by clathrin-coated pits has been a subject of great interest recently. Since many internalization signals on the cytoplasmic domains of proteins which are endocytosed through this mechanism share similar structural motifs, it is attractive to suggest that a common recognition protein is involved. The adaptor protein complex, AP2, has been proposed to be the "recruiter" protein (Pearse, 1988). AP2 is directly involved in the recruitment of clathrin to the membrane and potentiates the formation of clathrin structures. Early evidence that AP2 interacts with cytoplasmic domains of receptors targeted for internalization came from work that described its specific association with an affinity matrix column composed of the internalization signal-containing cytoplasmic domains of either the low-density lipoprotein receptor (LDLR) or the mannose-6-phosphate receptor (M6PR) (Glickman et al., 1989; Pearse, 1988). The ability of the cytoplasmic domains of internalizing receptors (LDLR, M6PR, immunoglobulin receptor) to elute AP2 from the affinity column lent support to the idea that AP2 was a common component of the endocytic mechanism. Co-immunoprecipitation studies revealed a strong interaction between AP2 and activated epidermal growth factor receptor (EGFR) (Nesterov et al., 1995; Sorkin and Carpenter, 1993). In contrast, recent evidence suggested that the high affinity binding of AP2 to the EGFR was not necessary for efficient internalization of the EGFR (Nesterov et al., 1995; Sorkin et al., 1996). No measurable binding of AP2 to the cytoplasmic domain of the TfR has been demonstrated, though interactions between the tetra peptide internalization

sequence of the TfR and the μ subunit of AP2 have been detected using the two hybrid system and surface plasmon resonance (Ohno et al., 1995).

Generation of a cell line capable of overexpressing a single cycling receptor, specifically the TfR, should slow the endocytosis of other receptors if indeed there is a common protein involved with the coated pit recognition mechanism. The present set of experiments demonstrate that overexpression of the TfR does saturate some rate limiting step or component of its own endocytosis. In uninduced fTfR20-2 cells, as well as the parent tTA HeLa cell line, an average of 1.50×10^5 TfRs are on the cell surface and Tf is internalized at a rate of 0.39 Tf molecules per surface TfR per minute. When fTfR20-2 cells are induced, the surface TfR population increases to between 2×10^6 and 4×10^6 surface TfRs per cell, and a decreased rate of as low as 0.10 Tf molecules internalized per surface TfR per minute is measured.

Our finding that the endocytosis of a constitutively cycling receptor can be saturated is at odds with the proposal that only the endocytosis of triggered receptors are saturable. The idea that the endocytosis of constitutively recycling receptors is not saturable at high receptor concentrations arose from two observations. First, two reports about coated pit formation in cells with high numbers of TfR have been published. Iacopetta and co-workers used electron microscopy to demonstrate that high TfR densities led to an increase in the number of coated pits forming at the plasma membrane (Iacopetta et al., 1988). Miller and co-workers presented evidence that although an increase in flat clathrin lattice area was apparent in cells with high numbers of TfRs, the actual number of coated pits remained constant (Miller et al., 1991). These results imply that receptors recruit AP2 and clathrin to the plasma membrane. We do not detect any

redistribution of AP2 from the cytosol to the plasma membrane or any increase in AP2 or clathrin concentrations (data not shown) in cells under conditions that we see comparable numbers of TfRs (the induction of TfR is 10-20 fold and the rate of ^{125}I-Tf uptake/ surface TfR is decreased four fold). Second, Rothenberger and co-workers reported that no changes in the rate of ^{125}I-Tf endocytosis per surface TfR was detected in mouse cells expressing large numbers of human TfRs (4.6 x 10^6) versus lower numbers of TfRs (~1.5 x 10^5), although the uptake rate of ^{59}Fe per TfR decreased (Rothenberger et al., 1987). They concluded that exocytosis rather than endocytosis slowed at high TfR numbers. No changes of the rate in exocytosis of the TfR were detected in our system (see figure 9 in supplementary section of this chapter). Our results imply that TfRs do not actively recruit adaptor proteins to the plasma membrane but rather the TfRs diffuse to, or aggregate in pre-existing coated pits, and that this process can be saturated.

The reasons why our results differ from the previous results are not totally obvious. The method that we used to observe the association of AP2 with the plasma membrane has been shown by several groups to reflect an accurate AP2 distribution (Chang et al., 1991; Mahaffey et al., 1990; Moore et al., 1987). The method of measuring ^{125}I-Tf uptake differs from that of Rothenberger and co-workers (Rothenberger et al., 1987) in that our assay does not include prebinding Tf at 4°C before allowing uptake to occur at 37°C. Perhaps, the initial rates of endocytosis that Rothenberger and co-workers measured could have been affected by the cells recovering from a low temperature block of endocytosis.

The idea that the cytoplasmic domains of receptors do not directly recruit AP2 to the plasma membrane is consistent with recent work by Santini and co-workers (Santini

and Keen, 1996). Using an immobilized antigen for IgE Fc receptors, they were able to show receptor activation without internalization, which lead to an aggregation of receptors at the exposed surface. No discernible difference in the level or distribution of either clathrin or AP2 between activated and inactive receptors was detected. Together with our data, this suggests that AP2 recruitment and coated pit formation are regulated independently of receptor concentrations.

The lack of competition that we have seen between the EGFR and the TfR for endocytosis is consistent with earlier experiments. Using A431 cells that normally express a high density of many types of receptors, Wiley demonstrated that internalization of the EGFR could be saturated (Wiley, 1988). Since the EGFR is a triggered receptor, it does not cluster into coated pits until it binds EGF. By modifying the amount of EGF given to cells, the effective concentration of active EGFR in the coated pits could be increased, and the rate of internalization measured. Though saturation of the endocytosis of the EGFR was obvious, the rate of TfR internalization was unaffected. Studies of Lamaze and colleagues demonstrated that endocytosis of EGFR and TfR have different biochemical requirements (Lamaze et al., 1993).

In summary, we show that overexpression of a single recycling receptor can overwhelm endocytosis such that the rate of internalization per receptor decreases. This saturation, which is dependent on the surface receptor number, reflects a limiting factor for TfR internalization. Second, we show that the rate of internalization for the EGFR does not change with the induction of TfR expression. This supports the idea that there are at least two different rate limiting steps in the endocytosis of these two receptors.

Finally, we show that the overall distribution and expression level of AP2 remains the same, independent of receptor number.

The system described in this paper provides a valuable resource for examining specific interactions of the cytoplasmic domains of internalizing receptors, with members of the endocytic machinery. In the case of the TfR, we are studying a single tyrosine-based sorting motif. This type of signal has been shown to be important for the internalization of a number of other membrane proteins, such as TGN38 (Wilde et al., 1994), the asialoglycoprotein receptor (Fuhrer et al., 1991), the low-density lipoprotein receptor (Bansal and Gierasch, 1991; Chen et al., 1990), and VSV-G (Thomas and Roth, 1994). Many membrane proteins have multiple possible endocytic signals, such as the insulin receptor beta-subunit which has two tyrosine-based signals in tandem (Backer et al., 1992). The cation-dependent mannose-6-phosphate receptor has a tyrosine-based as well as a di-leucine-based sorting motif (Canfield et al., 1991; Johnson et al., 1990), as do the EGFR (Chang et al., 1993) and glucose-4 transporter (Verhey et al., 1995). Often, the signals which are being utilized are difficult to dissect using site-directed mutagenesis. Internalization-deficient mutants could result from an indirect effect of destroying tertiary or quaternary structure of the entire cytoplasmic domain, rather than from the desired effect of altering the endocytic signal. Our system allows us to saturate the system with one receptor, and quantitatively look for competition *in vivo* with unaltered endogenous receptors.

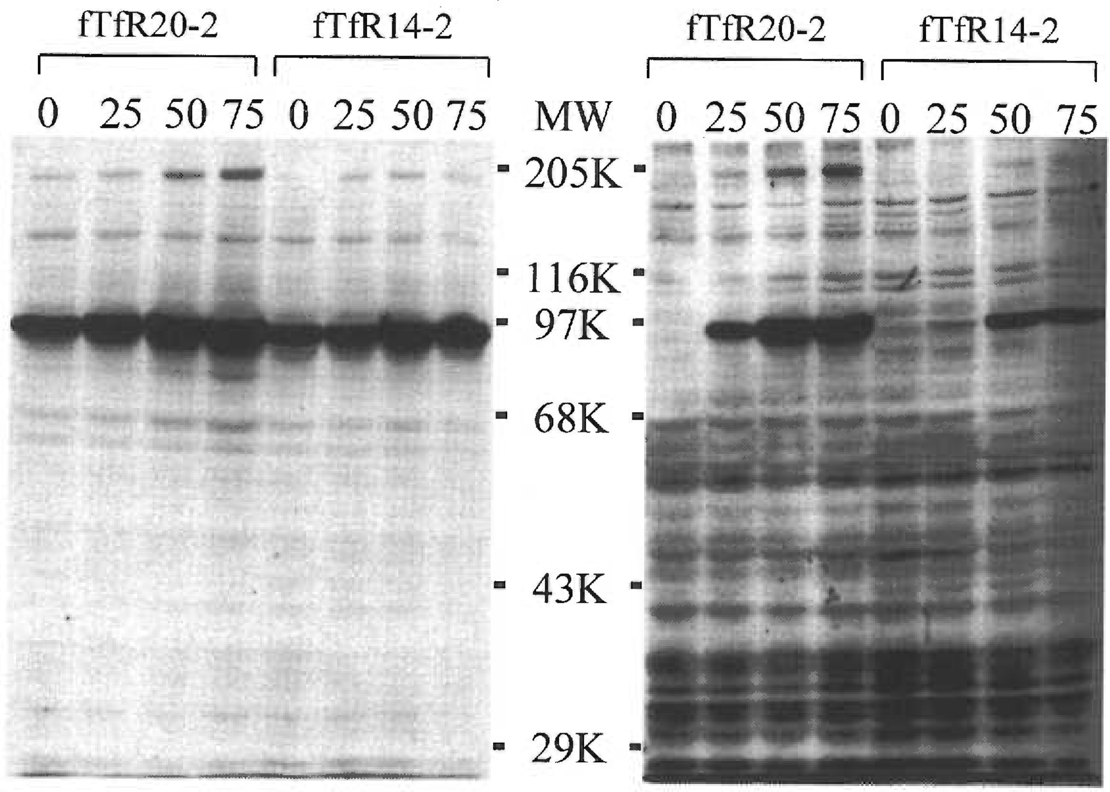

Figure 1. *Time course of induction for two individual tetracycline-responsive clones.* Two clones (fTfR14-2 and fTfR20-2) were grown in the absence of tetracycline to turn on the synthesis of the TfR for the number of hours indicated. Cells were solubilized and duplicate lysates were run on SDS-acrylamide (8%) gels. Gels were run under reducing conditions, transferred to nitrocellulose, and immunodetected with either sheep anti-TfR primary and horse radish peroxidase-conjugated swine anti-goat secondary antibodies (left panel), or rabbit anti-FLAG primary and horse radish peroxidase-conjugated goat anti-rabbit secondary antibodies (right panel).

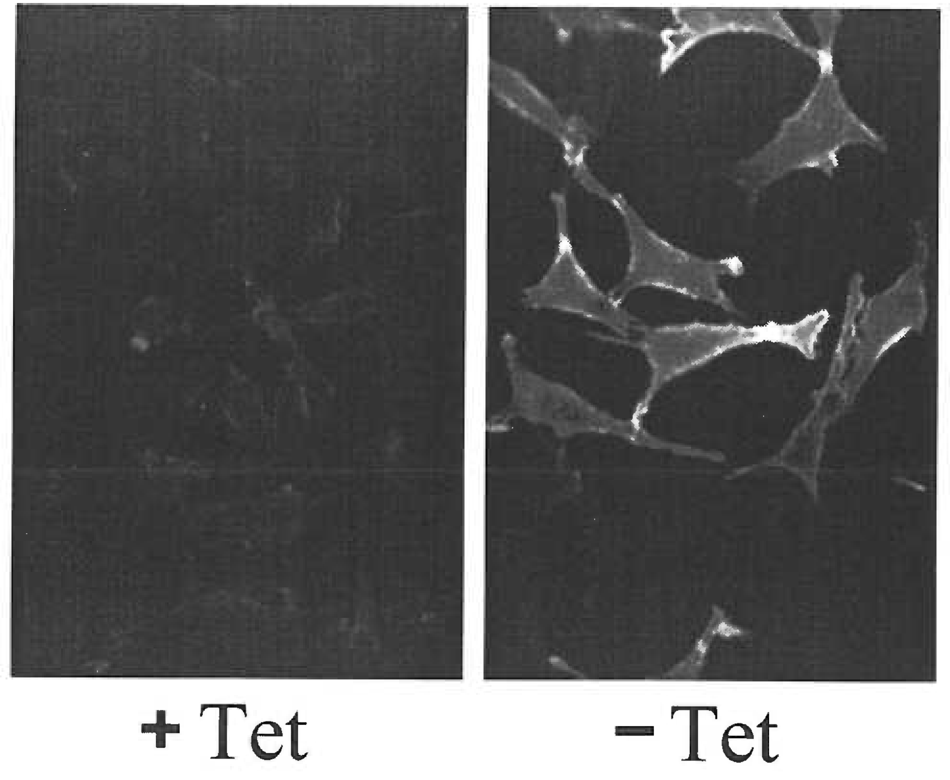

Figure 2. *Immunofluorescent detection of cell surface TfR in induced and uninduced fTfR20-2 cells.* fTfR20-2 cells were grown on coverslips in the presence (uninduced) or absence (induced) of 2 mg/ml tetracycline in the media as indicated, for 96 hours. Cells were fixed in 3% paraformaldehyde, and sequentially incubated with sheep anti-TfR primary and FITC-labeled swine anti-goat secondary antibodies.

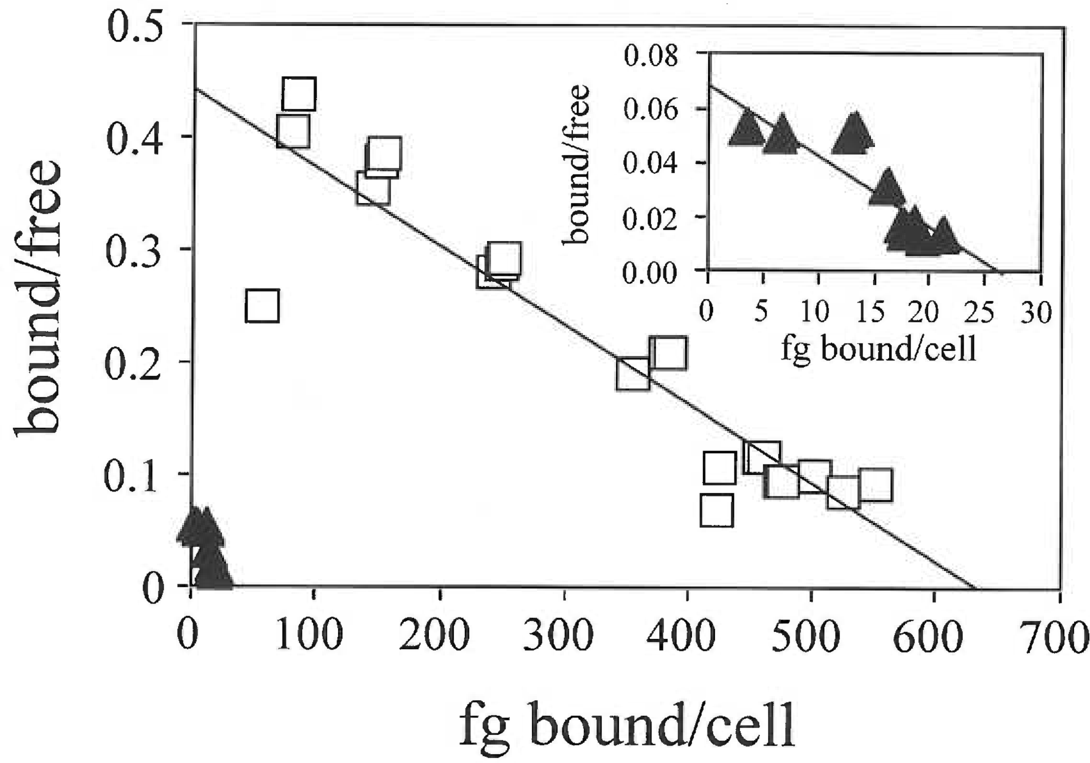

Figure 3. *Scatchard analysis of ^{125}I-Tf binding on fTfR20-2 cells.* Cells incubated with a range of ^{125}I-Tf concentrations (0.5 to 12 nM for uninduced cells, 5 to 120 nM for induced cells) for 90 minutes at 4°C, were washed four times with PBS, solubilized and counted in a gamma counter. Results were plotted as amount bound/free vs bound. Calculated values for the dissociation constant (KDa) for uninduced (closed triangles) and induced (open squares) fTfR20-2 cells were 3.0 nM and 25.8 nM respectively. Inset shows same plot for uninduced cells at a different scale for clarity.

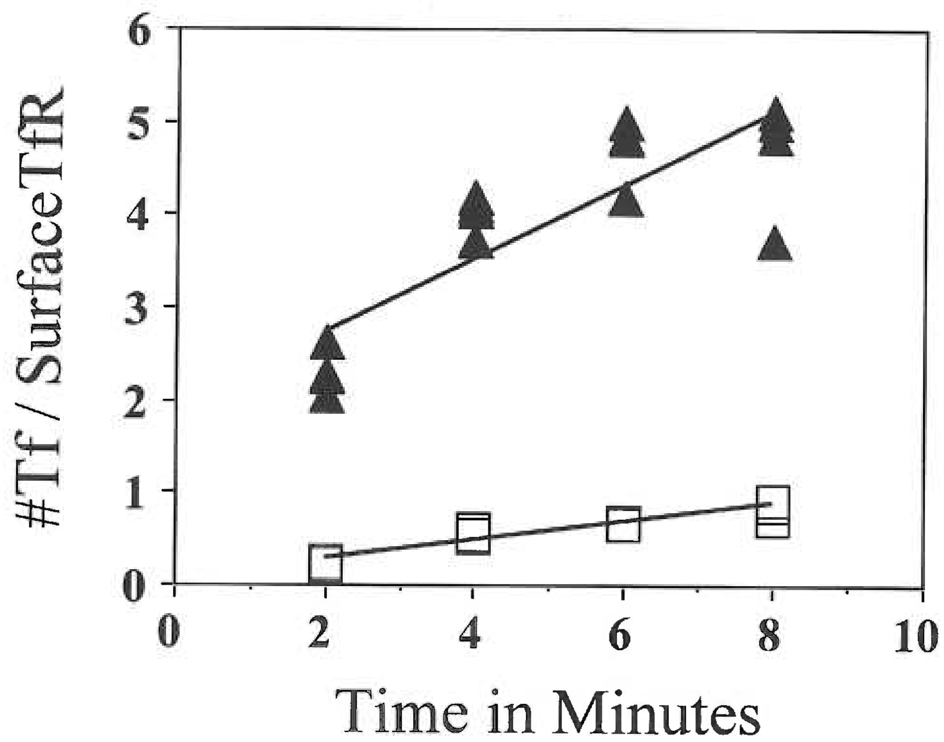

Figure 4. *Comparison of the rates of internalization of Tf for induced and uninduced cells.* Induced (passaged for at least 7 days in the absence of tetracycline) and uninduced fTfR20-2 cells were exposed to 210 nM ^{125}I-Tf for 2, 4, 6, or 8 minutes at 37°C. Surface ^{125}I-Tf was stripped with acid wash (see methods) prior to solubilization and were counted in a gamma counter. Simultaneous ^{125}I-Tf bindings were performed on cells for 90 minutes at 4°C to determine the number of receptors expressed on the cell surface. Results are plotted as a calculated measure of Tf molecules taken up per surface TfR. The calculated rate of uptake and receptor number for uninduced cells (closed triangles) is 0.39 Tf/TfR/min and 1.37 x 10^5 TfR/cell surface, respectively. For induced cells (open squares), these values are 0.10 Tf/TfR/min and 1.62 x 10^6 TfR/cell surface.

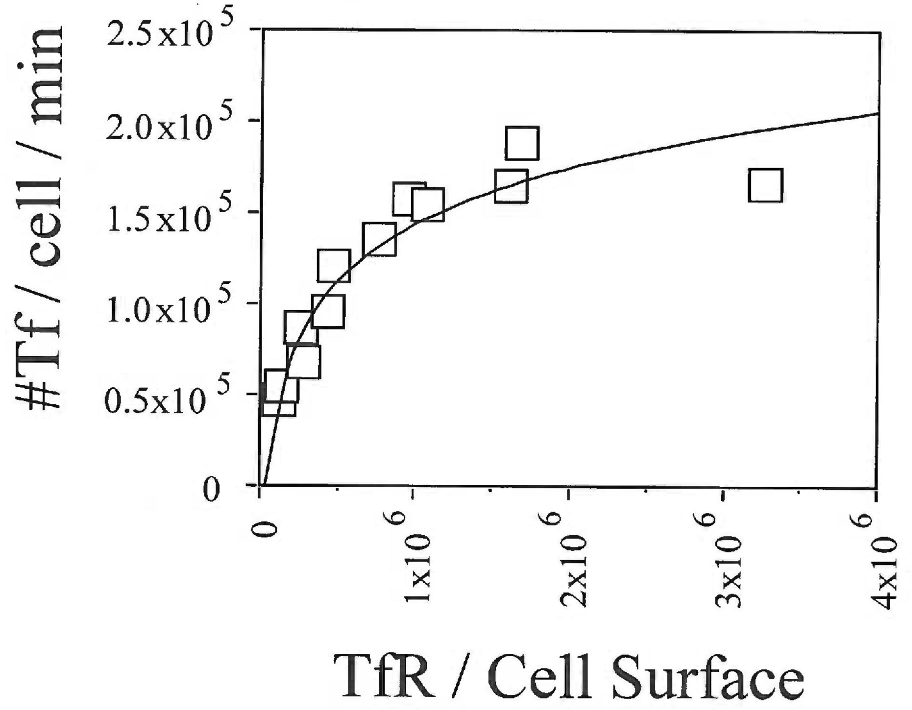

Figure 5. *Saturation of TfR endocytosis relative to receptor number.* fTfR20-2 cells were induced to overexpress TfR and then repressed with tetracycline for 0, 12, 24, 48, and 72 hrs, to generate a range of TfR numbers. Individual uptake assays were performed as described previously on each set of repressed cells, as well as uninduced cells. Each point represents a single uptake assay from which the receptor number and internalization rate were determined. The results from two experiments are plotted together.

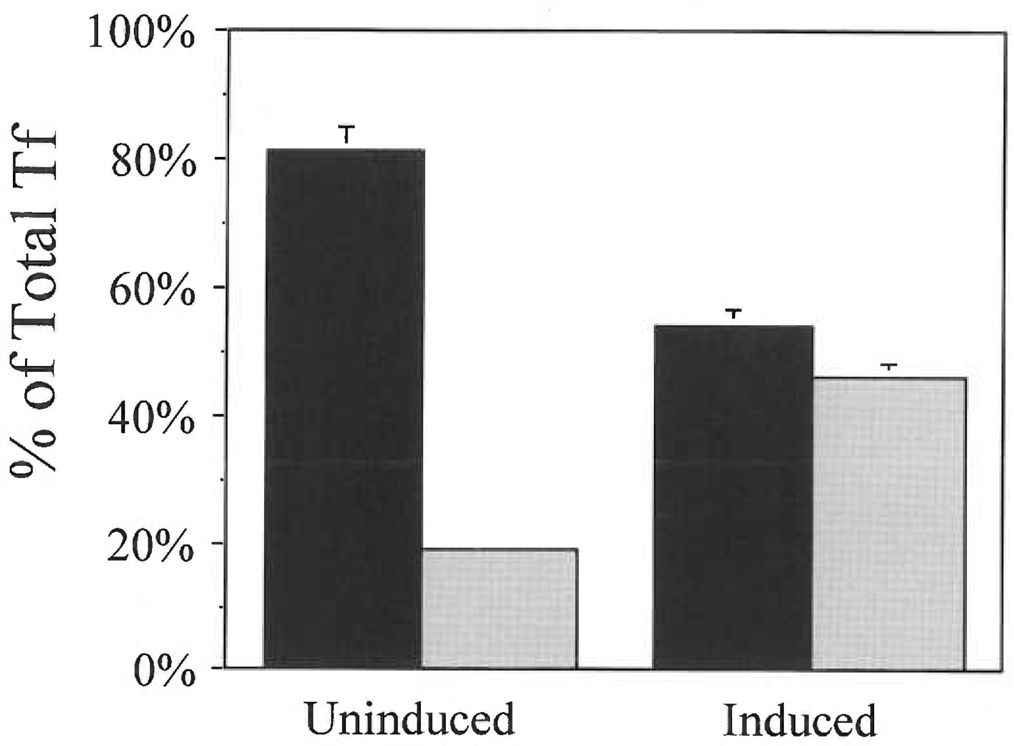

Figure 6. *Distribution of TfR in induced and uninduced cells.* Cell surface TfRs are determined by incubating cells with media containing 210 nM ^{125}I-Tf for 90 minutes at 4°C, solublizing and counting. For internal and total receptor determinations, cells are first pre-equilibrated with 210 nM ^{125}I-Tf for 30 minutes at 37°C and 5% CO_2. To determine internal TfR number, cells are either solubilized immediately (total) or washed with acid wash prior to solubilization and counting (internal). Internal (solid) and surface (open) TfRs are plotted as a percent of the total TfRs in the cell. Error bars are determined from the mean of two experiments.

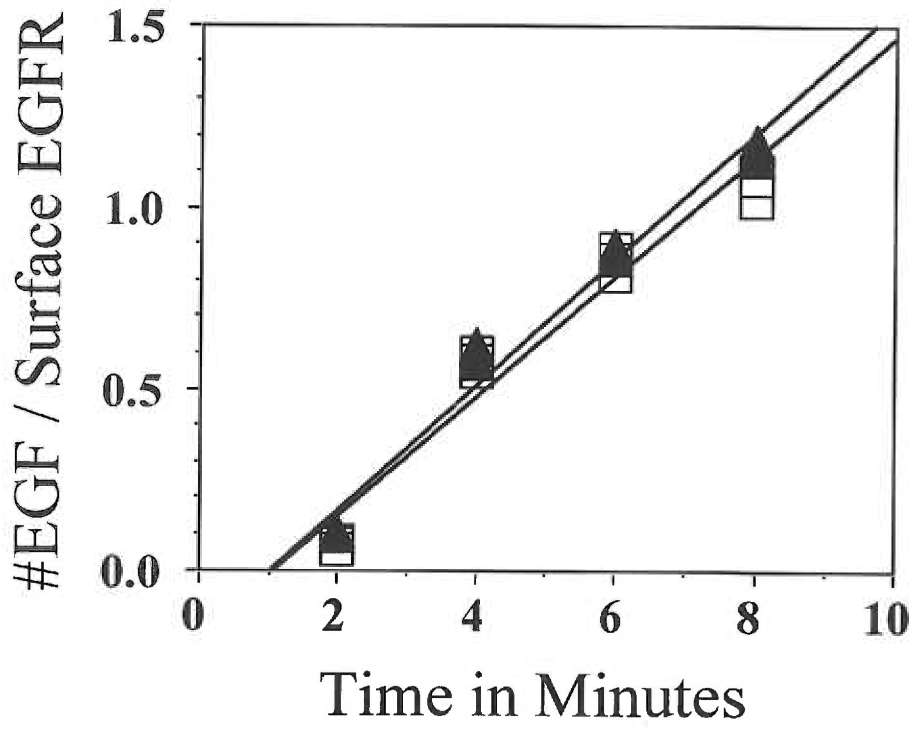

Figure 7. *Comparison of the Rates of Internalization of EGF for Induced and Uninduced cells.* Experiment is identical to Figure 4, except that uninduced (closed triangles) and induced (open squares) cells were incubated with 2.5 nM ^{125}I-EGF instead of ^{125}I-Tf. The calculated rate of EGF uptake, EGFR number, and TfR number for uninduced cells is 0.17 EGF/EGFR/min, 3.00 x 10^4 EGFR/cell surface and 1.80 x 10^5 TfR/cell surface, respectively. For induced cells, these values are 0.16 EGF/EGFR/min, 3.00 x 10^4 EGFR/cell surface, and 1.87 x 10^6 TfR/cell surface.

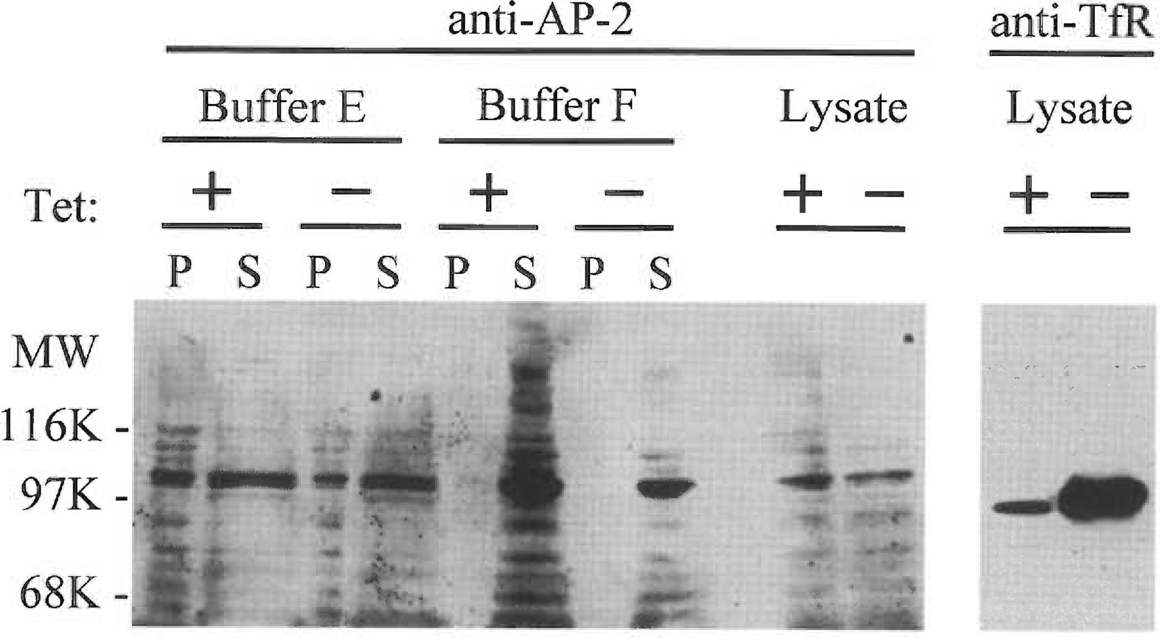

Figure 8. *Lack of AP2 redistribution to cellular membranes.* Uninduced (Tet +) and induced (Tet -) fTfR20-2 were freeze fractured to expose the cytoplasmic face of the lipid bilayer. Fractured cells were treated with either Buffer E (AP2 intact) or Buffer F (AP2 removed) prior to scraping wells to remove attached membranes. The pelleted membranes (P), supernatants (S) and whole cell lysates (L) representing equal portions of sample were run on reducing SDS acrylamide (8%) gels, transferred to nitrocellulose and probed with polyclonal antibodies to either AP2 or TfR. This experiment was performed by Frank Green.

Supplementary Data to Chapter 1

Rationale. When the fTfR is overexpressed, a decrease in the rate of ^{125}I-Tf internalization per TfR can be measured. There are two possible explanations for the observed effect: a) endocytosis is less efficient due to saturation of the endocytic mechanism, or b) exocytosis is slowed by an unrelated mechanism. One way to disprove the second possibility is to measure and compare the rates of exocytosis in uninduced and induced cells. Here, I demonstrate that the rate of exocytosis remains a constant, independant of the number of fTfR expressed on the cell surface.

Method. The release assay procedure was modified from a protocol by McGraw et. al. (McGraw and Maxfield, 1990). fTfR20-2 cells, grown in the presence (uninduced) or absence (induced) of 2 μg/ml tetracycline, were incubated with 210 nM ^{125}I-Tf at 37°C and 5% CO_2 for 2 hours. After performing a mild strip of the surface proteins with a mild acid buffer (500 mM NaCl, 50 mM MES, pH 5.0) for 2 minutes at 37°C, the cells were washed several times in final wash (150 mM NaCl, 20 mM HEPES, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, pH 7.4) containing a block mixture (17 μg/ml cold Tf and 100 μM desferrioxamine), to prevent rebinding of released ^{125}I-Tf. Treated cells were then incubated in 1 ml of DMEM containing block mixture for 2, 4, 6, or 8 minutes at 37°C and 5% CO_2. Plates were placed onto ice, and samples for each of three conditions collected sequentially as shown:

EFFLUX - Media was removed by pipetting, and the cells rinsed once with 1 ml of final wash. Media and final wash were pooled and counted in a gamma counter.

SURFACE - Cells were incubated in 1 ml of acid wash (500 mM NaCl, 500 mM Acetic Acid, pH 2.0) for 3 minutes. The acid wash ws removed by pipetting and the cells were washed once with cold final wash. Both washes were pooled and counted in a gamma counter.

INTERNAL - The remaining surface stripped and washed cells were solublized in 2 ml of solublization solution (0.1% Triton X-100, 0.1% NaOH) and counted in a gamma counter.

Results and Discussion. The effect of fTfR overexpression on the rate of exocytosis was measured by performing a release assay on fTfR20-2 cells that were grown in either the presence (uninduced) or absence (induced) of 2 μg/ml tetracycline. Samples were collected that represented ^{125}I-Tf as an amount released into the media (efflux), or as an amount remaining inside the cells (internal). The result of plotting the efflux of ^{125}I-Tf into the media as a percentage of the total is shown in Figure 9a. Despite a nearly 10-fold increase in the total number of TfRs expressed on the cell surface (data not shown), no significant effect on the efflux curve is observed. The same is true if exocytosis is measured as a rate of ^{125}I-Tf loss from the inside of cells (Figure 9b). These results suggest that exocytosis is not affected by overexpression of fTfR, and that the reduction of internalization is due to a saturated endocytic mechanism.

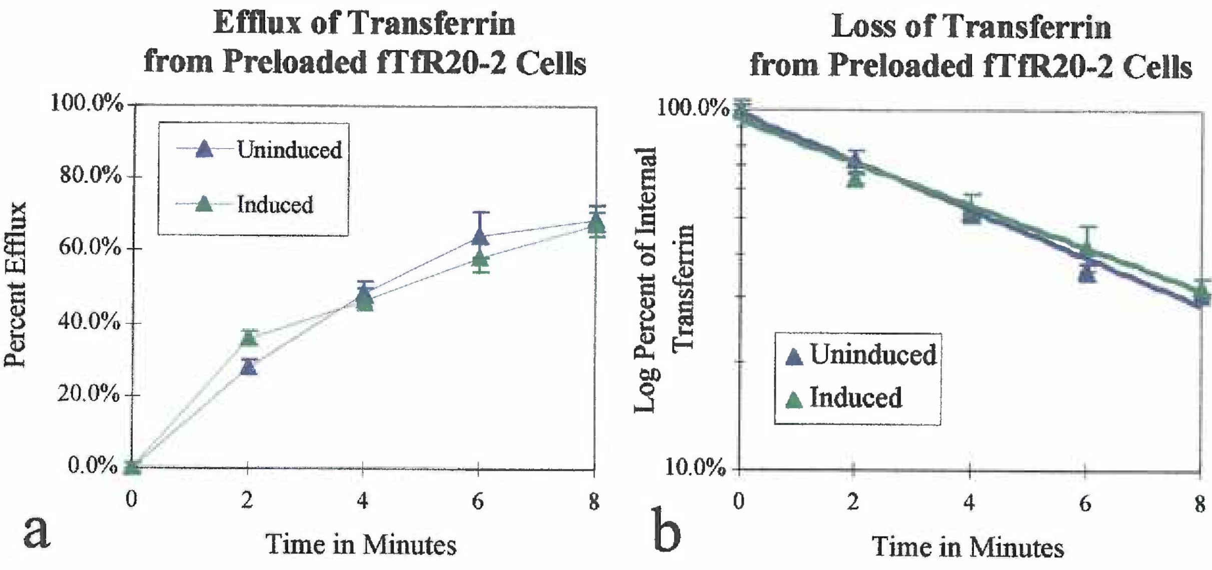

Figure 9. *The Rate of Exocytosis Remains Unchanged During Overexpression of the fTfR.* Release assays were performed on fTfR20-2 cells that were grown in the presence (uninduced) or absence (induced) of 2 μg/ml tetracycline. a) Media from fTfR20-2 cells preloaded with ^{125}I-Tf was collected at several timepoints and counted in a gamma counter. Points were plotted as a percentage of the total ^{125}I-Tf in the efflux relative to the surface and internal samples. b) fTfR20-2 cells used in the efflux assay were solublized and counted in a gamma counter for the presence of ^{125}I-Tf remaining inside. The results are plotted as a percentage of the total ^{125}I-Tf relative to the efflux, surface, and internal samples.

CHAPTER 2

Distinct Saturable Pathways for the Endocytosis of Different Tyrosine Motifs

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SUMMARY

Endocytosis of surface proteins through clathrin-coated pits requires an internalization signal in the cytoplasmic domain. Two types of internalization signal have been described: one requiring a tyrosine as the critical residue (tyrosine-based motif), and the other consisting of either two consecutive leucines or an isoleucine and leucine (di-leucine motif). Though it seems that these signals are necessary and sufficient for endocytic targeting, the mechanism of recognition is not well understood. To examine this question, tetracycline-repressible cell lines were used to overexpress one of several receptors bearing a tyrosine-based internalization signal. By measuring the rates of endocytosis for either the overexpressed receptor, or that of other endogenous receptors, we were able to show that the endocytosis of identical receptors could be saturated, but a complete lack of competition exists between the transferrin receptor (TfR), the low-density lipoprotein receptor (LDLR), and the epidermal growth factor receptor (EGFR). Overexpression of any one of these receptors resulted in its redistribution toward the cell surface, implying that entry into coated-pits is limited. During high levels of TfR expression however, a significant increase in the amount of surface Lamp1, but not LDLR, EGFR, or Lamp2, is detected. This suggests that Lamp1 and TfR compete for the same endocytic sites. Together, these results support the idea that there are at least three distinct saturable components involved in clathrin-mediated endocytosis.

INTRODUCTION

Cells selectively internalize specific surface receptors and their ligands through receptor-mediated endocytosis. This process begins when receptors, targeted for endocytosis, are selectively sequestered into specialized structures on the plasma membrane, termed clathrin-coated pits. These sites of rapid endocytosis are responsible for the internalization of a large variety of surface receptors and proteins. The endocytic machinery is able to recognize receptors destined for internalization through short stretches of amino acids in their cytoplasmic domains containing specific targeting information (for reviews, see (Kirchhausen et al., 1997; Marks et al., ; Trowbridge et al., 1993)). At least two types of internalization signals have been described: the tyrosine-based motif and the di-leucine based motif. Probably the more thoroughly examined of these is the tyrosine-based motif, which consists of a sequence of four to six amino acids, specifically containing a tyrosine that is crucial for proper endocytic targeting (Canfield et al., 1991; Chen et al., 1990; Collawn et al., 1993; Fuhrer et al., 1991; Johnson and Kornfeld, 1992; Roth et al., 1986). This tyrosine, which usually resides in a YXX∅ or NPXY motif (where X is any amino acid and ∅ is a hydrophobic residue), has been shown to capable of forming a tight turn in secondary structure (Bansal and Gierasch, 1991; Collawn et al., 1990; Eberle et al., 1991; Pytowski et al., 1995).

The mechanism behind the sequestration of surface receptors in coated pits remains largely unknown. Some receptors, like the EGFR[1] and Insulin-R, require ligand binding before they are concentrated into clathrin-coated pits and internalized. Other receptors, including the transferrin receptor, LDLR, and ASGPR, are constitutively

recycled, and spend a majority of their cell surface time clustered in coated pits (reviewed in (Goldstein et al., 1985)). In both cases, sorting signals have been suggested to mediate interactions between the receptor and AP2 complexes present in the coated pit (Marks et al., 1996). These AP2 associations, which have been shown *in vitro* for a number of different receptors (Chang et al., 1991; Ohno et al., 1995; Pearse, 1985; Pearse, 1988), would serve either to trap receptors in clathrin-coated pits, or insure that they are present at the location where new clathrin-coated pit structures are being assembled.

In previous work, we have shown that overexpression of a Flag-epitope tagged version of the constitutively endocytosing TfR (fTfR) can lead to saturation of endocytosis for the TfR, but does not affect the rate of internalization of the triggered EGFR (Warren et al., 1997). This result supports in part work by Wiley in 1988 (Wiley, 1988) and suggests that these two receptors are being internalized by different mechanisms. To examine this further, we have developed two new tetracycline-repressible cell lines that can overexpress the LDLR or the EGFR. In this report, we show that both EGFR and LDLR can be expressed at sufficient levels to saturate their own endocytic mechanism, but do not alter the rate of internalization of each other or the transferrin receptor. When the TfR is overexpressed however, a significant and repeatable accumulation of Lamp1 on the plasma membrane is detected, with no change in the amount of surface Lamp2. We suggest that multiple distinct saturable components exist for clathrin-mediated endocytosis.

MATERIALS AND METHODS

Plasmids—The plasmids LDLR/pTZ1 and EGFR/pBS were kind gifts from David Russell at the University of Texas Southwestern Medical Center, and Steven H. Wiley at the University of Utah School of Medicine, respectively. The pAlter– plasmid was purchased from Promega.

Subcloning and Mutagenesis—Using Promega's Altered Sites *in vitro* Mutagenesis System, the parent plasmid, pAlter–, was mutagenized with the oligonucleotide, PAXbaI (5'-agt att cta gag tgt cac c-3'), which adds a second Xba I site to the end of the multiple cloning region, and the oligonucleotide AmpRep (5'-gtt gcc att gct gca ggc atc gtg gtg-3'), which repairs the damaged ampicillin resistance gene, which is in turn used for selection. The resulting plasmid, pAlterXX, was used as an intermediate vector for cloning receptor DNAs into the pUHD10-3 vector (Gossen and Bujard, 1992).

To create the LDLR/pUHD10-3 construct, the Hind III - Hind III fragment from LDLR/pTZ1 was cloned into the Hind III site of pAlterXX. The resulting LDLR/pAlterXX was then used to isolate the Xba I - Xba I fragment containing the LDLR sequence, and subsequently cloned into the Xba I site of pUHD10-3. The EGFR/pUHD10-3 construct was made by first cloning the Xba I - Sal I fragment from EGFR/pBS into pAlter– and then mutating the EGFR/pAlter– plasmid with oligonucleotides PAXba I and AmpRep as described above. This EGFR/pAlterXX plasmid was used to isolate a Xba I - Xba I fragment containing the EGFR sequence,

which was then cloned into the Xba I site of pUHD10-3. All mutageneses were confirmed by sequencing.

Generation of Cell Lines—The tetracycline-repressible LDLR21 and EGFR8 cell lines were created using protocols described previously (Warren et al., 1997). Briefly, 20 μg of plasmid containing the tetracycline-responsive promoter region and sequence for either the LDLR (LDLR/pUHD10-3) or EGFR (EGFR/pUHD10-3) were cotransfected into HeLa cells expressing the tTA fusion protein with 500 ng of plasmid conferring puromycin resistance (pBSPac). Transfected cells were selected in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone), 400 μg/ml G418 (Calbiochem), and 400 ng/ml puromycin (Sigma). Stable colonies were screened for highest receptor expression in the absence of tetracycline by Western blotting.

Antibodies—The monoclonal mouse αLDLR (C7) antibody was obtained from ascites from the C7 cell line available through ATCC. The purified monoclonal mouse αEGFR (538) antibody was purchased from Santa Cruz Biotechnology. The monoclonal mouse αLamp1 (H4A3) and mouse αLamp2 (H4B4) antibodies, developed by Dr. J. Thomas August and Dr. James E.K. Hildreth, were obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, under contract NO1-HD-7-3263 from the NICHD.

Immunodetection—Non-reducing SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (Williams and Enns, 1991). Markers

used on gels were purchased from Sigma, and included myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa). After transfer, the molecular weight marker lanes were stained with Ponceau S for detection.

Iodination—Transferrin (Intergen) and EGF (Gibco BRL) were labeled as described previously (Warren et al., 1997). Prior to labeling, monoclonal antibodies were purified from ascites by binding to a column of either Protein-A agarose from Sigma (C7 and 2G11) or Protein A/G+ agarose from Santa Cruz Biotechnology (H4A3 and H4B4), followed by washes with 10 column volumes of 100 mM Tris pH 8.0 and 10 column volumes of 10 mM Tris pH 8.0. Purified antibody was eluted with 100 mM glycine pH 3.0. Fractions were collected in tubes containing 1 M Tris pH 8.0 (to neutralize the acid), and protein content determined by OD_{280} measurement. Fractions were analyzed by SDS-PAGE and Coomassie blue staining to verify purity and concentration. Once purified, either C7 (720 μg), H4A3 (60 μg), or H4B4 (60 μg) was used in a 50-100 μl reaction of 225 mM sodium phosphate buffer (pH 7.0), 0.5 units of lactoperoxidase (Sigma), 0.5 to 1.0 mCi of carrier-free $Na^{125}I$ (DuPont NEN), and 0.003% H_2O_2. After 5 minutes at room temperature, 25-50 μl 2% blue dextran was added, and the protein was separated from unreacted $Na^{125}I$ on a 2-ml desalting column (Pierce). Purification and labeling of LDL was performed as described by Goldstein et. al. (Goldstein et al., 1983).

Surface Binding—Cells grown to 90% confluency (~10^6 cells per well) in 6-well plates, were washed on ice 2 times with cold wash medium (Dulbecco's modified Eagle's medium

buffered with 20 mM HEPES pH 7.4). Incubation mixes were prepared for either ^{125}I-Tf (35 nM ^{125}I-Tf ± 12.5 μM unlabeled Tf), ^{125}I-EGF (2.5 nM ^{125}I-EGF ± 250 nM unlabeled EGF), ^{125}I-C7 (21 nM ^{125}I-C7 ± 500 nM unlabeled C7), ^{125}I-H4A3 (5 nM ^{125}I-H4A3 ± 260 nM unlabeled H4A3) or ^{125}I-H4B4 (2.6 nM ^{125}I-H4B4 ± 430 nM unlabeled H4B4). Non-specific binding measurements were made by including unlabeled ligand or antibody in the incubation mixes as shown. All bindings were carried out on ice, rocking for 90 minutes, after which they were washed four times in cold final wash (150 mM NaCl, 20 mM HEPES pH 7.4, 1 mM $CaCl_2$, 5 mM KCl, and 1 mM $MgCl_2$), solublized in 1.5 ml of 0.1 N NaOH, 0.1% Triton X-100, and counted for 1-6 minutes in a gamma counter.

Uptake Protocol—The rate of uptake was determined as described previously for the ligands ^{125}I-Tf and ^{125}I-EGF (Warren et al., 1997). Concentrations of ^{125}I-labeled or unlabeled Tf, EGF, and C7 used in the uptake assay are identical to those described for the surface binding procedure described above. Since our normal acid stripping procedure was only about 30% efficient at removing surface bound C7 antibody, it was modified as follows: Instead of a 2 minute wash with 3 ml of Acid Wash (0.2 N Acetic Acid, 0.5 M NaCl), cells were incubated on ice for 5 minutes with 3 ml of 30 ug/ml pronase (Pierce). This stripping procedure removed at least 90% of all surface bound ^{125}I-C7, without affecting the final cell count per well. Internalization rates for all ligands are expressed as number of ^{125}I-labelled molecules internalized per surface receptor per minute.

Immuno-Electron Microscopy—Cell lines were fixed in 4% paraformaldehyde and 0.1% gluteraldehyde in 100 mM HEPES (pH 7.2) for 30 minutes at ambient temperature. The fixed cell pellets were rinsed with 100 mM HEPES, infused with polyvinylpyrrolidone and sucrose (Tokuyasu, 1989) and prepared for cryosectioning (Griffiths et al., 1984; Griffiths et al., 1983). Each section was immunolabeled with sheep anti-TfR IgG (Warren et al., 1997), followed by mouse anti-goat IgG (Jackson Research Labs), and goat anti-mouse IgG conjugated to 10-nm gold particles (Amersham). Controls included the substitution of primary antibody with irrelevant antibodies, normal sheep IgG, or PBS.

RESULTS

Competition Between "Like" Receptors. We have generated three cell lines which can be induced to overexpress different receptors possessing tyrosine-based internalization signals, allowing us to test whether these signals will compete with themselves and each other. Stable HeLa cell lines transfected with the tetracycline-repressible system were created to overexpress either the EGFR, the LDLR, or a Flag epitope-tagged TfR (fTfR). Western blots of positive stable clones selected from approximately 40 to 50 colonies show the degree to which the EGFR and LDLR can be induced. Clones LDLR21 (Fig. 1a) and EGFR8 (Fig. 1b) were chosen as the highest tetracycline-responsive expressors of LDLR and EGFR, respectively. Though EGFR8 cells can be maintained in the same growth media as fTfR20-2 cells, fully induced LDLR21 do not survive as well. This is probably due to an excessive and unregulated transport of lipid into the cell by the large population of LDLRs present on the cell surface. The appearance of induced LDLR21 cells supports this theory since numerous fatty vesicles, as determined by Oil Red-O staining (see figure 7 in supplementary section of this chapter), are present in the cytosol. In order to circumvent this problem, 10% delipidated fetal bovine serum (prepared as described in (Goldstein et al., 1983)) was used in lieu of normal serum in LDLR21 media. Though use of this media led to the upregulation of endogenous LDLR and TfR (and possibly others), all subsequent comparisons with the LDLR21 cells were done under identical serum conditions.

Characterization of the fTfR20-2 cells from previous studies showed that expression of fTfR as high as 20-fold over endogenous TfR, leads to significant

competition for endocytosis (Warren et al., 1997). To determine whether induction of the LDLR could saturate its own endocytosis, the rate of internalization for ^{125}I-labeled C7 antibody (mouse IgG_{2a} anti-LDLR) (Beisiegel et al., 1981) was examined in LDLR21 cells with differing degrees of LDLR expression. Brown and Goldstein had previously demonstrated that this antibody reacts with a 1:1 stoichiometry with the LDLR and have used it to measure the cycling of the LDLR (Beisiegel et al., 1981). Use of the C7 antibody instead of the receptor's natural ligand, LDL, circumvents difficulties associated with loss of LDL activity after radioactive labeling. Varying levels of LDLR expression were obtained by first growing several sets of cells in the absence of tetracycline to maximal expression, and then adding tetracycline (2 μg/ml) to individual sets at different times before the experiment was performed (from 0 to 20 hours). For each set of cells, a surface binding and uptake assay were performed using ^{125}I-C7 to determine the surface LDLR number and rate of LDLR internalization, respectively. Our results show that across a 3.8-fold increase in the number of surface LDLRs (Fig. 2a), a corresponding 3.2-fold decrease in the rate of LDLR internalization is observed (Fig. 2b). For comparison, Davis and colleagues have shown that cells expressing LDLRs that are unable to undergo clathrin-mediated endocytosis due to a Tyr→Cys substitution in their internalization signal, internalize ^{125}I-LDL at a rate that is 5-fold slower than that seen with normal cells (Davis et al., 1986). This suggests a minimum rate of endocytosis and sets the lower limit for the effect of competition.

The competitive effect of EGFR overexpression was examined by performing a surface binding and uptake assay on EGFR8 cells, using ^{125}I-labeled EGF as the ligand, under conditions where all the EGFR would be triggered to endocytose. EGFR8 cells,

grown either in the presence (+) or absence (–) of 2 μg/ml tetracycline, show a 6.8-fold increase in surface expression of EGFR (Fig. 2c). At this level of expression, a corresponding 5-fold decrease in the rate of EGFR internalization is detected (Fig. 2d). This receptor, like the LDLR, competes with itself for endocytosis.

These results demonstrate that overexpression of either receptor results in a decrease in the efficiency (rate/receptor) of internalization of that receptor. If the different receptors were competing for binding to different epitopes of the same protein, then they should show similar saturation curves. The rates of endocytosis for each receptor was plotted against the cell surface number for the particular receptor to determine whether saturation of the pathway occurred with the same number of receptors (Fig. 2e). The resulting graph indicates that the limiting component in EGF endocytosis is much lower than LDLR and TfR, implying that they compete for different rate limiting molecules.

TfR and LDLR Do Not Compete with Each Other for Internalization. The TfR and LDLR share similar trafficking pathways: they internalize via clathrin-coated pits, and recycle to the plasma membrane after delivering their cargo to the early endosome. Each receptor contains a tyrosine-based sorting motif in the cytoplasmic domain, but differ in their orientation to the plasma membrane (see Table I). Since TfR and LDLR are under constitutive recycling, and both are capable of saturating their own endocytic pathway, we wanted to determine whether overexpression of one of these receptors could directly affect the rate of endocytosis of the other. fTfR20-2 cells grown in the presence (+) or absence (–) of 2 μg/ml tetracycline were used to measure TfR and LDLR surface binding

and rate of uptake of ^{125}I-C7. Even though fTfR is expressed at levels shown previously to be sufficient for saturation of TfR internalization (Warren et al., 1997), neither the number of LDLR on the cell surface (Fig. 3a), nor the rate of LDLR internalization (Fig. 3b) changes significantly. This suggests that the fTfR is saturating a different component of clathrin-mediated endocytosis than that of the LDLR.

To examine whether the same was true for the saturation of LDLR endocytosis, LDLR21 cells expressing variable amounts of LDLR on the surface (prepared as described above), were used to measure endogenous surface TfR number (Fig. 3c) and rate of ^{125}I-Tf internalization (Figure 3d). Even though LDLR numbers are at levels shown earlier to be sufficient to saturate its own endocytosis, neither the number of surface TfRs, nor the rate of TfR internalization changes significantly. This experiment confirms our results with the fTfR20-2 cell line, and strongly supports the idea that these two receptors are competing for different limiting components.

LDLR Overexpression Does Not Interfere with EGFR Internalization. Previously, we have shown that TfR does not compete with EGFR for endocytosis (Warren et al., 1997), and in this study demonstrate a lack of significant competition between the TfR and LDLR. To determine whether LDLR and EGFR compete for endocytosis, LDLR21 cells were grown in the presence (+) or absence (−) of 2 μg/ml tetracycline, and used to measure both the surface LDLR and EGFR number, as well as determine the rate of uptake of ^{125}I-EGF. The results show that a 7-fold increase in LDLR (Fig. 3e) does not significantly affect either the surface expression of EGFR, or its rate of internalization (Fig. 3f).

Saturation of Different Endocytic Pathways Causes a Redistribution of Different Surface Markers. Saturation of an endocytic pathway by overexpression of a receptor should result in the accumulation of the receptor at the rate-limiting step in the endocytic/recycling pathway. Previous studies of ours and others indicate that the rate-limiting step is at an early point in the pathway, since receptors accumulate on the cell surface rather than in endocytic compartments (Marks et al., 1996; Warren et al., 1997). To visualize this and to determine whether the limiting step was at the level of receptor clustering or endocytosis, frozen thin sections of fTfR20-2 cells grown in the presence or absence of tetracycline were immunolabeled with sheep anti-TfR antibody, followed by mouse anti-goat IgG, and then goat anti-mouse IgG conjugated to 10-nm gold. In the absence of fTfR expression (Figure 4a), endogenous TfR is present on the plasma membrane in small amounts. After three days (Figure 4b) or more than 7 days (Figure 4b - inset) in the absence of tetracycline, fTfR expression increases dramatically and greater numbers of total TfRs are present on the cell surface. The immunogold label is more homogeneously distributed along the plasma membrane compared to cells lacking fTfR expression. In addition, there is not a significant increase in the number of coated vesicles in these cells (data not shown). These results suggest that the limiting step during overexpression of fTfR is at the level of receptor aggregation.

If the TfR distribution shifts to the plasma membrane during endocytic saturation of this receptor, then it follows that other molecules competing for the same pathway of internalization should show a similar shift in equilibrium, detectable as an increase in surface population. We decided to look at the surface populations of the lysosomal

associated proteins Lamp1 and Lamp2 for several reasons. First of all, both of these proteins have very short cytoplasmic domains (see Table I) each containing a highly conserved tyrosine residue in a YXX∅ conformation (Fukuda, 1991; Fukuda et al., 1988). Since there are only a few amino acids exposed to the cytoplasm, it is likely that the tyrosine-motif is the only signal for internalization. Second, substitution of the critical tyrosine residue from Lamp1 with an alanine residue completely abolishes clathrin-mediated endocytosis of this molecule (Williams and Fukuda, 1990), indicating that the Lamp1 tyrosine motif is indeed an endocytosis signal. Though the tyrosine motif from Lamp2 has not yet been demonstrated as a functional internalization signal, the striking similarity of its cytoplasmic domain to that of Lamp1 suggests that it is. Finally, overexpression of a Tac-antigen reporter chimera containing a tyrosine-based internalization signal leads to an accumulation of Lamp1 as well as Lamp2 on the cell surface (Marks et al., 1996), implying that both of these proteins can compete for internalization.

fTfR20-2 or LDLR21 cells grown in the presence (+) or absence (−) of tetracycline were used to measure binding at 4°C of ^{125}I-labeled monoclonal antibodies to either Lamp1 or Lamp2. In fTfR20-2 cells overexpressing the fTfR (Fig. 5a), we see a significant increase in the surface Lamp1 population when fTfR is expressed 8-fold above endogenous levels, but no significant change in the numbers of plasma membrane Lamp2 (Fig. 5b). Overexpression of LDLR (Fig. 5c) however, does not affect the number of surface Lamp1 or Lamp2 protein (Fig. 5d). Detection of surface Lamp2 in both cell lines was minimal, making exact quantitation difficult, but the results were the same each time

tested. These results imply that Lamp1 is competing with the fTfR for endocytosis, and may in fact utilize an identical pathway.

DISCUSSION

Even though receptors bearing tyrosine-based internalization signals are not necessarily destined for the same location in the cell, they are all initially dependent on clustering into clathrin-coated pits for efficient endocytosis. The mechanisms responsible for capturing these proteins may not be the same for each type of signal, but are most likely available in every coated pit. Two lines of evidence support this theory. First, double-label morphological studies have demonstrated colocalization of individual ligands to the same coated pit for EGF and LDL (Carpentier et al., 1982), EGF and Tf (Dickson et al., 1983; Hanover et al., 1984), Tf and ASGP (Neutra et al., 1985; Stoorvogel et al., 1987), Tf and α2-macroglobulin (Yamashiro et al., 1984), LDL and α2-macroglobulin (Via et al., 1982), EGF and α2-macroglobulin (Willingham et al., 1983), and EGF and insulin (Maxfield et al., 1978). More recent evidence shows that a heterogeneous population of receptors can be sedimented with a single horseradish peroxidase (HRP) conjugated ligand using 3,3'-diaminobenzidine (DAB) density shift analysis (Stoorvogel et al., 1989; Stoorvogel et al., 1987). The results showed that HRP/Tf/TfR-containing vesicles copurified with the ASGPR and M6PR. Together with the double-label studies, these results demonstrate that EGFR, LDLR, TfR, ASGPR, Insulin-R, and M6PR all utilize the same coated pits during endocytosis.

Recognition of plasma membrane proteins bearing tyrosine-based internalization signals by a common set of proteins at the clathrin-coated pit is an attractive mechanism by which these molecules can be targeted for internalization. This idea originally led a number of labs to try and identify proteins that were interacting with the internalization

signal. Results obtained with affinity chromatography (Beltzer, 1991; Glickman et al., 1989; Pearse, 1988; Sosa et al., 1993) and co-immunoprecipitation studies (Nesterov et al., 1995; Sorkin and Carpenter, 1993; Sorkin et al., 1996; Sorkin et al., 1995) demonstrated direct protein-protein interactions between a number of clathrin-coated pit targeted proteins and the clathrin-associated protein, AP2. More recently, Ohno and colleagues used the two-hybrid system to isolate the μ_2 subunit of the AP2 complex, using a triple-repeat of the tyrosine-containing sorting signal amino acid sequence SDYQRL from the TGN resident protein, TGN38, as bait (Ohno et al., 1995). Photoreactive peptides containing either this sequence or the Tyr→Ala mutated sequence SDAQRL have further defined the specificity of the μ_2 interaction to the tyrosine residue, and showed a dependence of the interaction on clathrin and phosphoinositides (Rapoport et al., 1997). Both the position and context of the internalization signal are determining factors in this interaction (Boll et al., 1996; Ohno et al., 1996). All of these findings suggest that AP2 may be important for either trapping clathrin-coated pit-targeted proteins, or providing the mechanism responsible for bringing them to the coated pit.

Though AP2 interactions might be necessary for the internalization of some proteins, it is clear that this is not the sole mechanism behind recognition of all tyrosine-based motifs. For example, results by independent labs indicate that if the EGFR is mutated such that it no longer has a high affinity site for AP2 binding, its rate of internalization is unaffected when compared to that of the wild type receptor (Nesterov et al., 1995; Sorkin et al., 1996). In addition, previous work by our lab and others show that although the TfR and EGFR both contain tyrosine-based internalization motifs, they are unable to saturate endocytosis for one another when overexpressed (Warren et al.,

1997; Wiley, 1988). This suggests that TfR and EGFR are saturating different limiting components of the endocytic mechanism.

In the current study, we have demonstrated that the TfR, LDLR, and EGFR saturate endocytosis at different receptor concentrations, and do not compete with each other for internalization. This was accomplished by saturating endocytosis with one of the receptors by overexpression, and examining the competitive effect on the rate of internalization of the others as endogenous receptors. We show that although overexpression of either LDLR or EGFR can saturate their respective endocytic mechanisms, no competition is evident between LDLR and TfR, or LDLR and EGFR. The implication, taken together with our previous results which show that the TfR and EGFR do not compete for internalization, is that each one of these receptors saturates at a different concentration and has a distinct rate-limiting component.

After demonstrating that the TfR, EGFR, and LDLR were not competing with each other for endocytosis, we wanted to see if any of these individual receptors could compete with other cell surface proteins bearing tyrosine-based internalization signals. Since saturation of its endocytic pathway leads to redistribution of the particular protein to the cell surface, this property was used as a criteria for competition for the same component in the endocytic pathway. By overexpressing one of the receptors, competition for endocytosis could be detected as an increase in surface population of the protein of interest. Cells overexpressing fTfR show a significant increase in the number of Lamp1 molecules on the cell surface, indicating that this protein is competing for the same limiting component or components as the TfR. This is in agreement with work by Marks and colleagues who showed that overexpression of a tyrosine-based sorting signal

causes a redistribution of TfR, Lamp1, and Lamp2 to the cell surface (Marks et al., 1996). We did not find however, that overexpression of TfR induced a redistribution of Lamp2 to the cell surface.

If all of these proteins are undergoing clathrin-mediated endocytosis, where does the difference in the abilities of the receptors to saturate their own, but not each others endocytosis arise? Though one pathway could be attributed to interactions with AP2, it is difficult to imagine that this complex becomes limiting at different concentrations of different endocytic signals. We hypothesize that intermediate connector proteins, most likely associated with the membrane, are responsible for the recognition and recruitment of a subset of tyrosine-based sorting signals (Fig. 6). In support of this theory, other proteins besides AP2 have been implicated in the recognition of internalization signals. The Eps15 protein, originally identified as a substrate for the EGFR tyrosine kinase domain, has been shown to associate with α-adaptin from the AP2 complex (Benmerah et al., 1996) and the light chain of clathrin (van Delft et al., 1997). This protein, which shows strong association with activated EGFR, may be the bridge necessary for EGFR recruitment to clathrin-coated pits. Another example is the β_2-adrenergic receptor which contains a tyrosine-based internalization motif (Valiquette et al., 1990) and requires the presence of β-arrestin or arrestin-3 for efficient internalization to occur (Goodman et al., 1996). Additionally, β-arrestin has been shown to interact directly with clathrin heavy chain (Goodman et al., 1997; Goodman et al., 1996; Krupnick et al., 1997), suggesting that it may play a role similar to AP2 in β_2-adrenergic receptor internalization.

Our model suggests that in addition to the AP2 complex, there may be a number of intermediate proteins, or sorting connectors, that can recognize and recruit receptors

destined for internalization. In untransfected cells (Fig. 6a), each type of connector would be available for recognition of a specific subset of tyrosine-based signals. As long as connectors are available, the endocytosis of receptors would remain unhindered. In cells overexpressing a single tyrosine motif, like that of the TfR (Fig. 6b), a depletion of sorting connectors would occur, and lead to a decreased efficiency of endocytosis of proteins carrying signals recognized by the same type of connector. This model predicts that saturation of one type of sorting connector by overexpression of one subset of tyrosine sorting signals would not hinder the recruitment of signals from different subsets. This model also predicts that receptors bearing multiple sorting signals could potentially saturate more than one type of connector, and affect the endocytic rate of more than one subset of signals. It is tempting to suggest that the sorting connectors may be playing a role in the recruitment of AP2 to the plasma membrane. The AP2 molecules could be recruited to the membrane directly via the connector proteins or only when the receptor/connector complex forms a high affinity site. In either case, the sorting connectors would be necessary to facilitate aggregation of receptors into clathrin-coated pits or at sites of clathrin-coated pit formation. Alternatively, the receptors could be interacting with different domains of the same protein. This explanation is unlikely to be true for the lack of competition between the EGFR and the TfR or the LDLR. The saturation of the rate limiting component for EGFR endocytosis occurs at a much lower concentration of receptors than either the TfR or the LDLR (Fig. 2e).

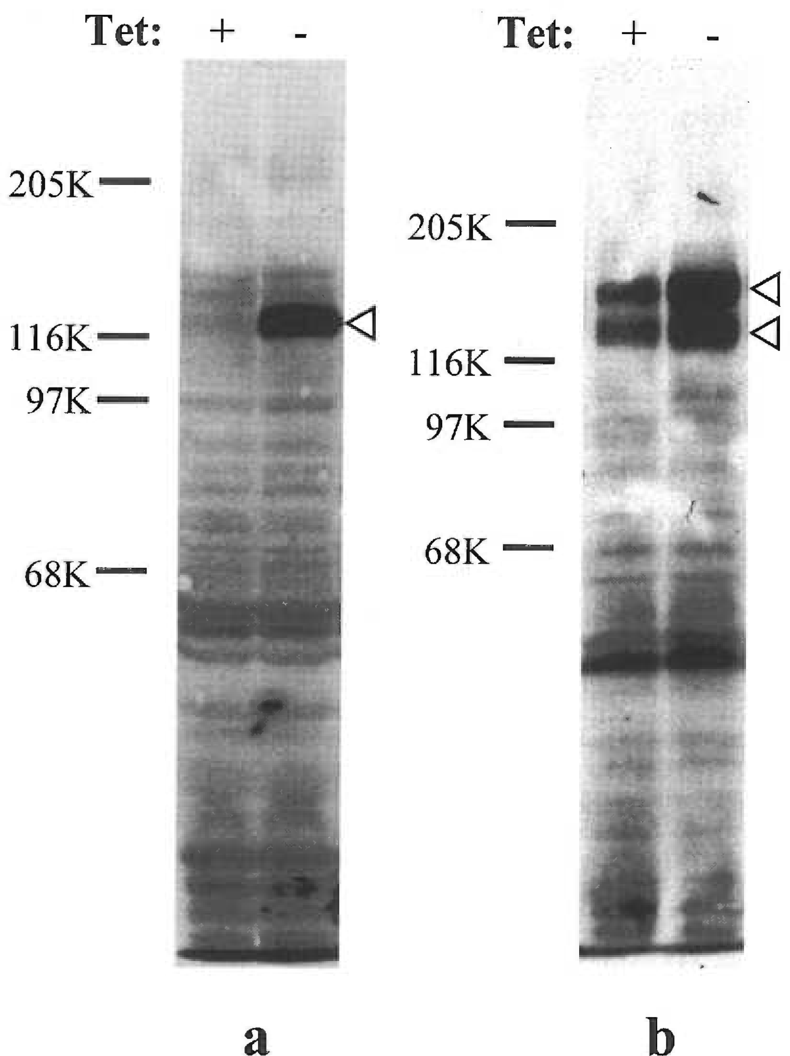


Figure 1. *Induction of LDLR and EGFR in tetracycline-repressible stable cells.* Cell lysates from either the LDLR21 (a) or EGFR8 (b) cell lines, grown in the presence or absence of tetracycline, were run on SDS-polyacrylamide (8%) gels under non-reducing conditions. After Western transfer to nitrocellulose, blots were immunodetected with C7 mouse anti-LDLR (a) or #538 mouse anti-EGFR (b), and horseradish peroxidase-conjugated secondary antibodies. Arrows indicate the position of the overexpressed LDLR (160 kDa) and EGFR (in the absence of protease inhibitors, runs as a doublet at 180/160 kDa). Endogenous LDLR in HeLa cells are difficult to detect by Western blotting techniques, thus explaining the lack of a visible band in the + lane of (a).

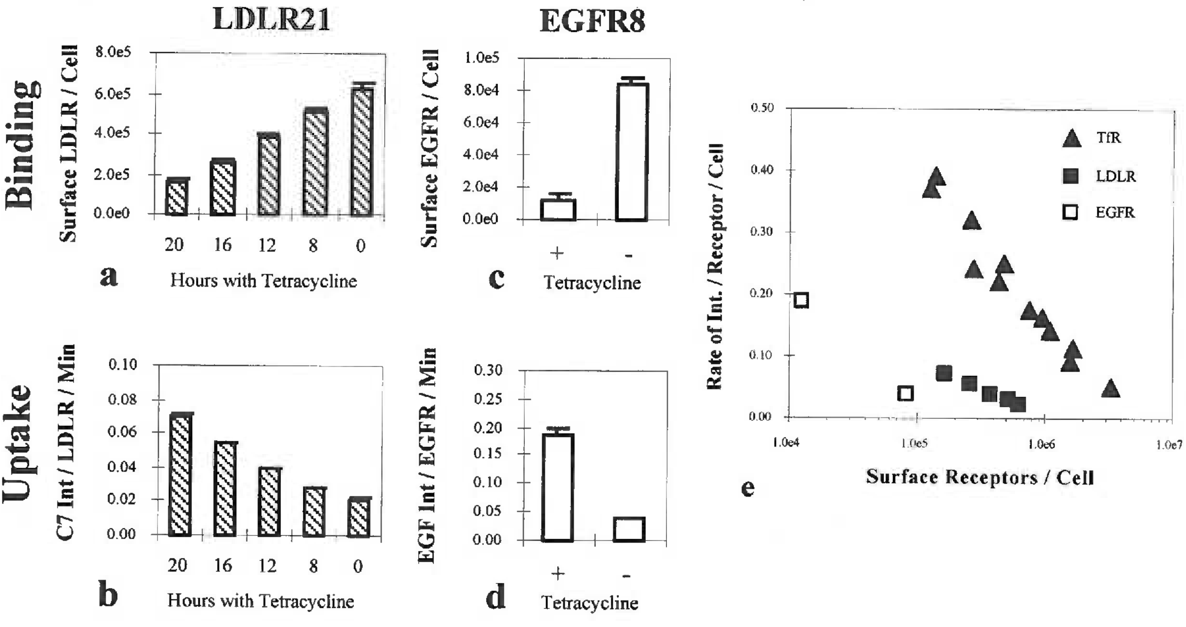


Figure 2. *Competition between "like" receptors.* The results from surface bindings (a and c) and uptake assays (b and d) are shown for two tet-repressible cell lines. LDLR21 cells maintained in the absence of tetracycline and thus expressing maximal amounts of LDLR, were repressed with 2 μg/ml tetracycline for decreasing periods of time (20 hrs, 16 hrs, 12 hrs, 8 hrs, 0 hrs) before the experiment was begun. A surface binding (a) and an uptake assay (b) were performed on cells from each time point, using saturating amounts of ^{125}I-labeled C7 (21 nM). ^{125}I-labeled EGF (2.5 nM) was used in a surface binding (c) and uptake assay (d) on EGFR8 cells grown in the presence (+) or absence (–) of

tetracycline. Two of six samples for each of the bindings and uptakes were done in the presence of either 500 nM unlabeled C7 antibody (a and b) or 250 nM unlabeled EGF (c and d) to provide nonspecific measurements. Error bars indicate the standard deviation of the mean of quadruplicate samples. The rate of internalization of the LDLR (solid squares) or EGFR (open squares) was plotted as a function of the number of each cell surface receptor (e). The data for the rate of TfR internalization and the surface TfR (solid triangles) was taken from Warren and coworkers internalization results (Warren et al., 1997), for comparison.

Table I. *Internalization signals*

Protein	Primary Localization	Sorting Signals
Tf Receptor	Early Endosome	N-terminus [19aa][YTRF][45aa][TMD]
LDL Receptor	Early Endosome	[TMD][12aa][FDNPVY][32aa] N-terminus
EGF Receptor	– EGF Plasma Membrane + EGF Lysosome	[TMD][Kinase][Multiple Signals?] N-terminus
Lamp1	Lysosome	[TMD][7aa][YQTI] N-terminus
Lamp2	Lysosome	[TMD][6aa][YEQF] N-terminus

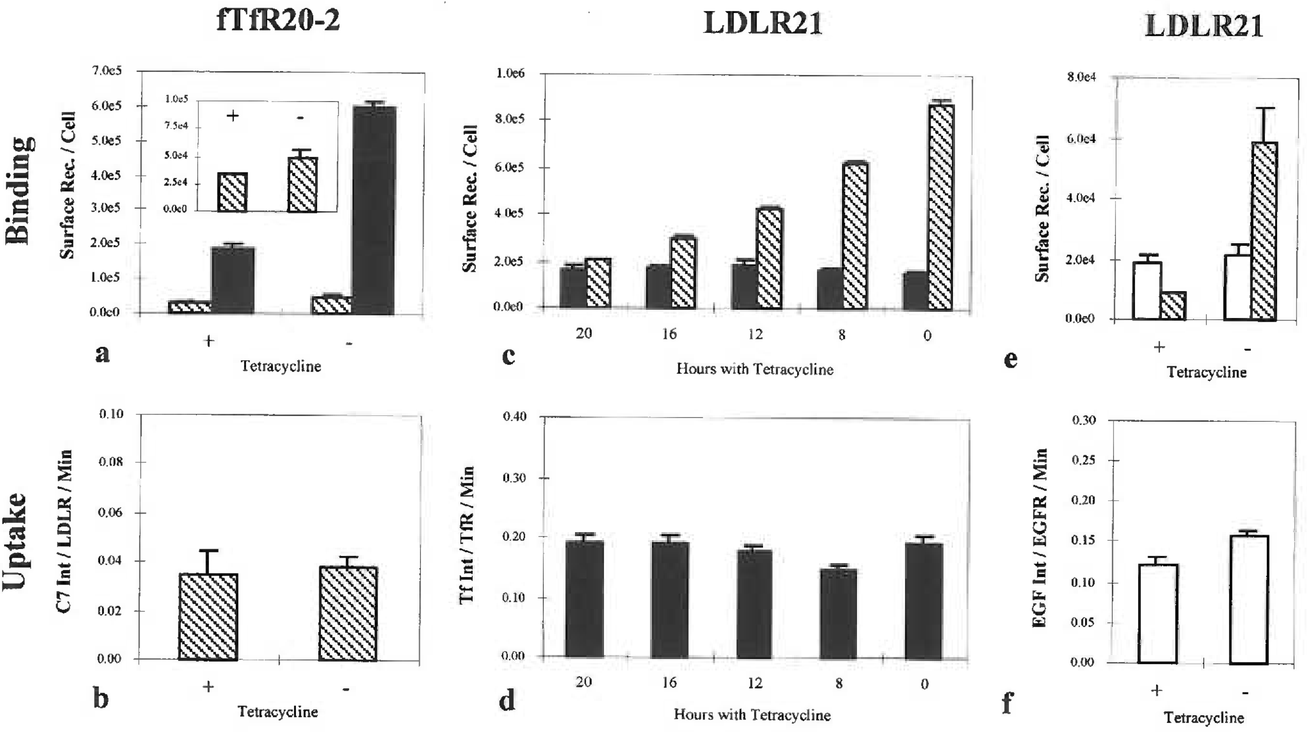


Figure 3. *Lack of competition between different receptors.* The results from surface bindings (a, c, and e) and uptake assays (b, d, and f) are shown for two tet-repressible cell lines. fTfR20-2 cells were grown in the presence (+) or absence (–) of 2 µg/ml tetracycline. A surface binding (a) was performed using either 21 nM ^{125}I-C7 (*cross-hatched bars* - see inset for clarity) or 35 nM ^{125}I-Tf (*solid bars*). ^{125}I-C7 was also used in an uptake assay (b) on the same set of cells. LDLR21 cells that were repressed for 0 to 20 hours, as described in Figure 2, were used to measure surface bindings (c) with either 21 nM ^{125}I-C7 (*cross-hatched bars*) or 35 nM ^{125}I-Tf (*solid bars*). ^{125}I-Tf was also used in an uptake assay (d) on the same set of cells. Surface bindings were also performed on LDLR21 cells grown in the presence (+) or absence (–) of 2 µg/ml tetracycline (e), using 2.5 nM ^{125}I-EGF (*white bars*) or 10 nM ^{125}I-LDL (*cross-hatched bars*). An uptake assay

(f) was performed on the same set of cells using 21 nM ^{125}I-EGF. Addition of 500 nM unlabeled C7, 100 nM LDL, 12.5 μM unlabeled Tf or 250 nM unlabeled EGF to two of six samples for each set were included in the respective incubation mixes for nonspecific binding and uptake measurements. Error bars indicate the standard deviation of the mean of quadruplicate samples. (Note: Though the rate of Tf-internalization in untransfected HeLa cells expressing endogenous numbers of surface TfRs is usually around 0.30 to 0.35 Tf/cell/min, the lower measured rate of 0.15 to 0.20 Tf/cell/min in LDLR21 cells (d) is most likely due to growth of cells in media supplemented with delipidated fetal bovine serum, which is necessary for the survival of LDLR21 cells.)

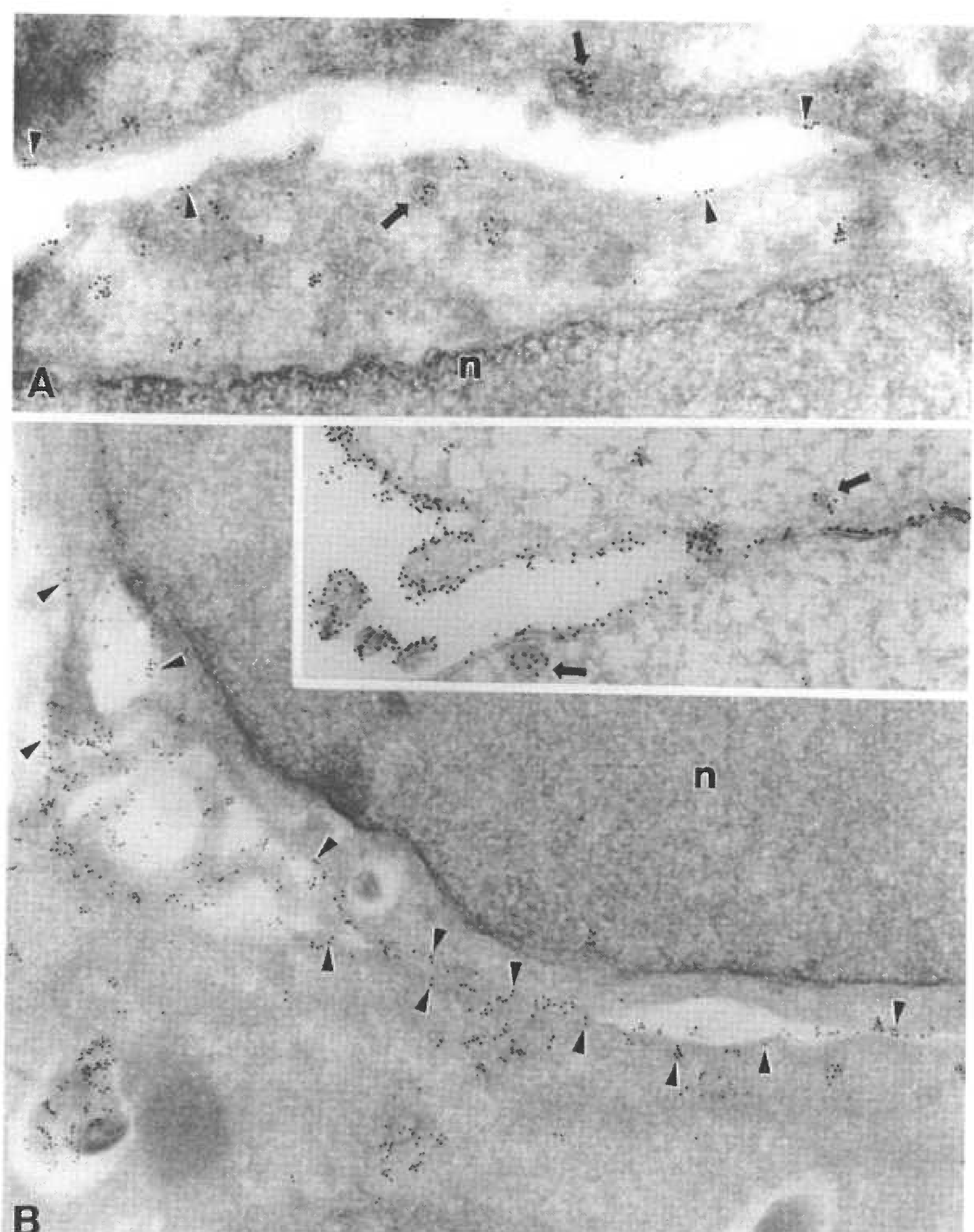

A
n
B
n

Figure 4. *Distribution of TfR in frozen thin sections of fTfR20-2 cells detected by immunogold labeling.* fTfR20-2 cells grown in the presence of 2 μg/ml tetracycline (A), grown without tetracycline for 3 days (B), or grown without tetracycline for more than a week (B - inset). Arrowheads indicate label along the plasma membranes; arrows indicate label within coated vesicles. n, nucleus. (A), original magnification × 50,000; (B), original magnification × 40,000; inset, original magnification × 50,000. Immunodetection and electron microscopy was performed by Paula Stenberg, Ph.D.

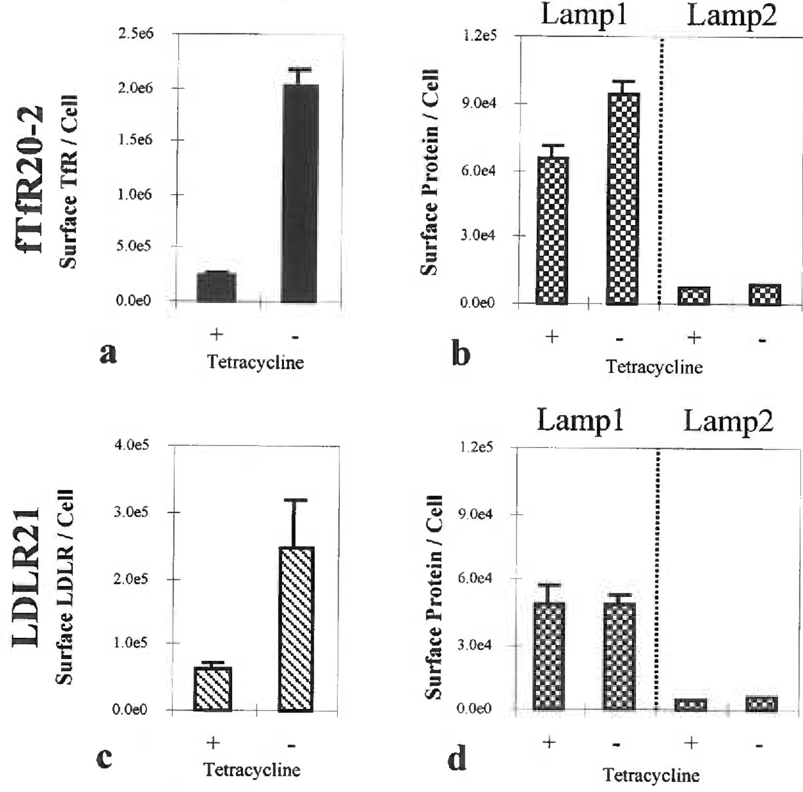
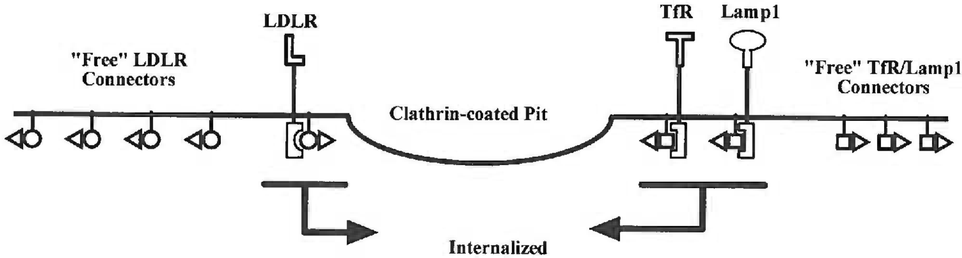


Figure 5. *Surface redistribution of proteins bearing tyrosine-based motifs.* Either fTfR20-2 cells (a), or LDLR21 cells (b) grown in the presence or absence of 2 μg/ml tetracycline were used in surface binding assays for overexpressed receptors (left panels) and three other proteins targeted for internalization (right panels). Surface TfR and LDLR were determined as described previously. Determination of surface number for the other proteins of interest were made by incubating cells for 90 minutes at 4°C with labeled antibodies for Lamp1 (5 nM ^{125}I-H4A3 ± 260 nM unlabeled H4A3) or Lamp2 (2.6 nM ^{125}I-H4B4 ± 430 nM unlabeled H4B4). Error bars represent the standard deviation of the mean from several experiments.

a) Untransfected cells



b) Cells overexpressing TfR

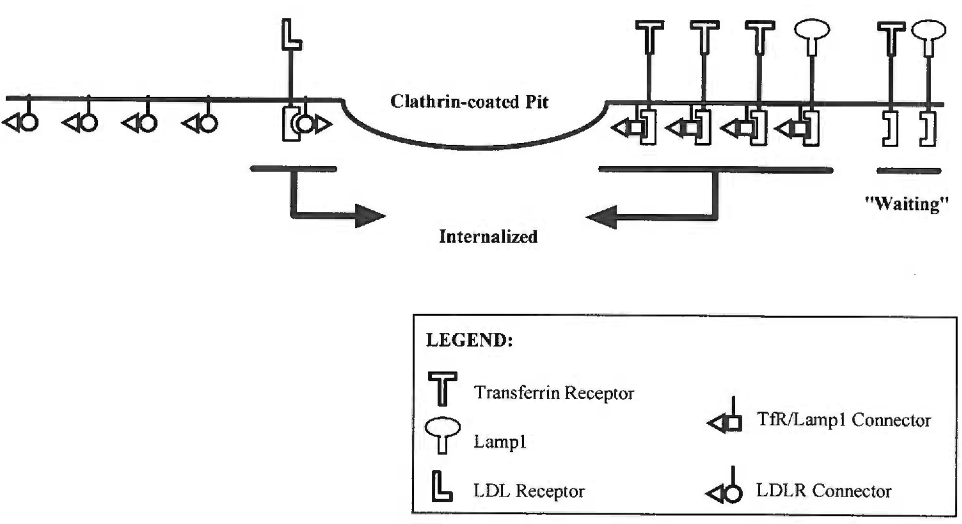


Figure 6. *Model depicting the role of sorting connectors in clathrin-mediated endocytosis.* (a) In untransfected cells, sorting connectors for LDLR and TfR/Lamp1 are in excess, and available to recruit receptors to the coated pit. (b) Cells overexpressing the TfR deplete the available TfR/Lamp1 sorting connector pool. The competition for TfR/Lamp1 connectors leads to an accumulation of both TfR and Lamp1 protein on the surface, "waiting" for free sorting connectors. Endocytosis of the LDLR continues unhindered, since its sorting connector pool is unchanged.

Supplementary Data to Chapter 2

Rationale. Two sets of experiments are described in this supplement to Chapter 2. The first experiment demonstrates the functionality of overexpressed LDLR by showing an accumulation of fatty vesicles in induced cells. High levels of LDLR eventually leads to cell death, a problem that is overcome by growing cells in a medium that is supplemented with lipid-depleted fetal bovine serum.

The second set of experiments was performed to show that endocytic competition by the TfR occurs through an intact internalization signal. Previous demonstration that overexpression of the TfR leads to an overall reduction in the rate of internalization for itself suggests that some component of the endocytic machinery is present in limiting amounts. The assumption is that the limiting component is interacting directly through an intact internalization signal necessary for efficient receptor endocytosis. By overexpressing the TfR in a form that lacks the extracellular transferrin binding domain, and contains either the normal YTRF internalization signal or an internalization defective mutant signal, ATRA, we can determine whether the signal itself is directly responsible for saturation of the endocytic machinery.

Method for Figure 7. LDLR21 cells were grown on coverslips in the presence or absence of 2 μg/ml tetracycline. Growth medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) containing either 10% fetal bovine serum (Hyclone), or 10% lipid-depleted fetal bovine serum (prepared as described in (Goldstein et al., 1983)).

The Oil Red O staining procedure was modified from the protocol described in (Ramirez-Zacarias et al., 1992). Oil Red O stain was prepared by adding 50 mg of Oil Red O powder (Matheson Curtis & Bell) to 10 ml of isopropanol, and mixing at room temperature for 15 minutes. Excess debris was removed by spinning at ~3000 RPM for 10 minutes, and filtering through a 0.2 μm filter. Immediately before use, 3 ml of stain was diluted with 2 ml water.

To stain for fatty vesicles, cells were removed from the growth media, washed twice in phosphate buffered saline (PBS), and fixed for 15 minutes at room temperature with a 3% paraformaldehyde solution. Fixed cells were washed three times with PBS, and immersed in an Oil Red-O stain (described above) for 10 minutes at room temperature. The fixed/stained cells were wased three times with distilled water to remove excess stain, and mounted for viewing with a microscope.

Method for Figure 8. Two constructs were created in the tetracycline-responsive promoter parent plasmid (pUHD10-3). The 150Flag/pUHD10-3 construct was made by performing polymerase chain reaction (PCR) on the full length TfR cDNA contained within the pAlter- plasmid from Promega (TfR/pAlter-) using the oligonucleotides EcoRIBeg (5'-ct tct gtg tgg gaa ttc aga atg atg-3'), which inserts an EcoRI site before the start codon, and 150FlagBam (5'-atc gga tcc tca ttt atc atc atc atc ttt ata gtc att ttc att cag cag-3'), which inserts the eight amino acid Flag-epitope and a termination codon immediately after amino acid number 150 in the TfR. The resulting PCR product was isolated and purified using Qiagen PCR preps, restriction digested with EcoRI and BamHI, and ligated into the respective sites of the pUHD10-3 parent plasmid.

Construction of the 150AAFlag/pUHD10-3 plasmid involved two steps. First, two separate PCR reactions were set up with TfR/pAlter-. One reaction included the oligonucleotides EcoRIBeg (see above) and PROM19 (5'-tga caa tgg ttc tcc acc-3'), which matches the region of the TfR just prior to Tyr20. The other reaction used A20A23Two (5'-gga gaa cca ttg tca gca aca cgg gca agc ctg gct cgg caa-3'), which modifies Tyr20 and Phe23 to alanines, and 150FlagBam (see above). Next, the PCR products from the two reactions were combined and run in a new PCR reaction. The resulting PCR product, identical to 150Flag described above except for two alanine changes, was then isolated, digested, and ligated as described above for the 150Flag/pUHD10-3 construct. Inserts were verified by nucleotide sequencing.

To generate the stable cell lines, 20 μg of each plasmid were cotransfected into HeLa cells expressing the tTA fusion protein with 500 ng of plasmid conferring puromycin resistance (pBSPac). Transfected cells were selected in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone), 400 μg/ml G418 (Calbiochem), and 400 ng/ml puromycin (Sigma). Stable colonies were screened for highest level of expression in the absence of tetracycline by Western blotting.

To analyze expression levels, whole cell lysates were run on 12% SDS-polyacrylamide gels under reducing conditions, and transferred to nitrocellulose. Western blots were probed with mouse monoclonal primary antibodies to either the TfR cytoplasmic domain (H68.4 from Zymed) or the Flag-epitope (M2 from Sigma), followed by horseradish peroxidase coupled goat anti mouse secondary antibodies.

Transferrin labeling and uptake assays are as described previously (Warren et al., 1997).

Results and Discussion. Overexpression of LDLR in LDLR21 cells is noticably detrimental to cell viability after more than a week. These cells take on a grainy appearance consisting of a large accumulation of vesicles in the cytoplasm that are not found in normal cells. We predicted that these vesicles represented the increased uptake of fatty molecules resulting from unregulated transport of lipid across the plasma membrane from the now large population of surface LDLR. The nature of these vesicles was tested by using the hydrophobic lipid stain Oil Red O, using a procedure described in (Ramirez-Zacarias et al., 1992). In uninduced LDLR21 cells, only a very small amount of red vesicle staining is seen (figure 7a), indicating the baseline level of fatty vesicles. However, when LDLR21 cells are grown in the absence of tetracycline, and LDLRs are overexpressed, a large accumulation of red-staining vesicles is seen clustered in the cytoplasm (figure 7b). Cells maintained in this condition do not survive for longer than a few weeks. If overexpressing cells are instead grown in a growth medium that is supplemented with 10% lipid-depleted fetal bovine serum, the cells do not show an accumulation of red-staining vesicles (figure 7c), and can survive for much longer periods of time. This provided us with a means to overexpress the LDLR without a loss of cell viability. All experiments performed on the LDLR21 cell line, described in the body of the text for Chapter 2, utilized the lipid-deficient serum to maintain cell viability. To normalize all uptake rate comparisons, lipid-deficient serum supplemented media was used for uninduced, as well as induced LDLR21 cells.

To determine if TfR endocytic competition by overexpression was specifically due to its tyrosine-based internalization signal, two stable tetracycline-responsive HeLa

cell lines were generated. The plasmids TfR150Flag/pUHD10-3 (150Flag) and TfR150AAFlag/pUHD10-3 (150AAFlag), were used to create stable cell lines using a protocol as described previously (Warren et al., 1997). The 150Flag sequence represents the TfR with a stop codon inserted at amino acid position 150, and includes the amino terminal cytoplasmic domain, transmembrane domain, and approximately 60 amino acids of the ectodomain. The 150AAFlag sequence is identical to the 150Flag sequence, except that the YTRF internalization signal is mutated to the internalization defective sequence ATRA. The resulting cell lines, 150Flag and 150AAFlag, were grown in the presence or absence of 2 μg/ml tetracycline for 3-4 days prior to measurement of internalization rate by transferrin uptake assays. Levels of expression of each of the two proteins was determined by SDS-PAGE and western analysis of cell lysates (Figure 8a). Western immunodetection using an anti-TfR monoclonal antibody shows no expression of either 150Flag (lane 1) or 150AAFlag (lane 2) in cells grown in the presence of tetracycline. 150Flag and 150AAFlag cells grown in the absence of tetracycline, however, show protein expression detectable as a doublet at around 22000 kDa (lanes 3 and 4, respectively). Flag-epitope immunodetection of cell lysates from 150Flag (lane 5) and 150AAFlag (lane 6) grown in the absence of tetracycline, indicate that the upper band of the doublet is the full length protein. The lower band most likely represents a degraded fragment.

Uptake rate measurements for the 150Flag cell line indicate a moderate decrease in the rate of endocytosis for the endogenous, full-length TfR (Figure 8b, gray bars) as high levels of expression of the 150Flag protein are induced in the absence of tetracycline. This is in accord with our previous data that overexpression of the TfR can saturate the

endocytic mechanism (Warren et al., 1997). In contrast, high expression levels of the 150AAFlag protein has very little effect on the rate of TfR internalization (Figure 8b - white bars).

In conclusion, these results suggest that the YTRF internalization signal is necessary for endocytic competition by overexpression, and implies a role for the TfR internalization signal to bind limiting components of the endocytic apparatus.

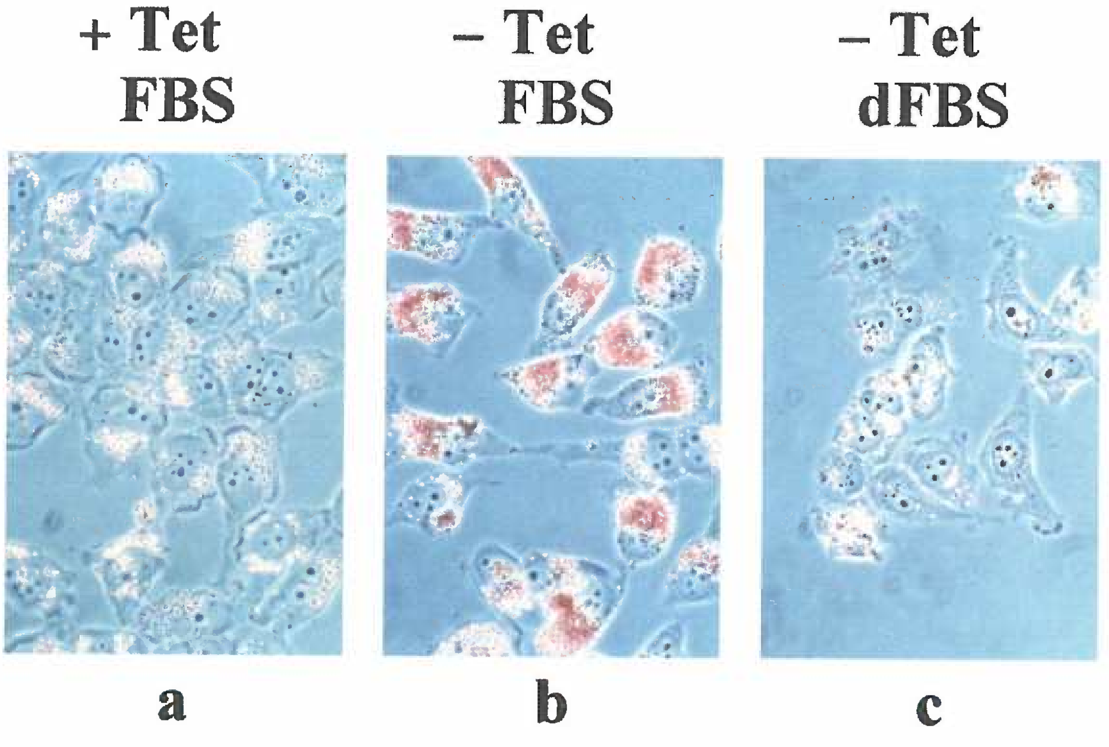


Figure 7. *Overexpression of LDLR leads to an accumulation of fatty vesicles.* LDLR21 cells were grown on coverslips in the presence (a) or absence (b and c) of 2 μg/ml tetracycline. Growth medium consisted of DMEM containing either 10% normal fetal bovine serum (a and b), or 10% lipid-depleted fetal bovine serum (c). All cells on coverslips were fixed in 3% paraformaldehyde, and stained with a 3 mg/ml Oil Red-O stain in isopropanol and water (3:2 ratio).

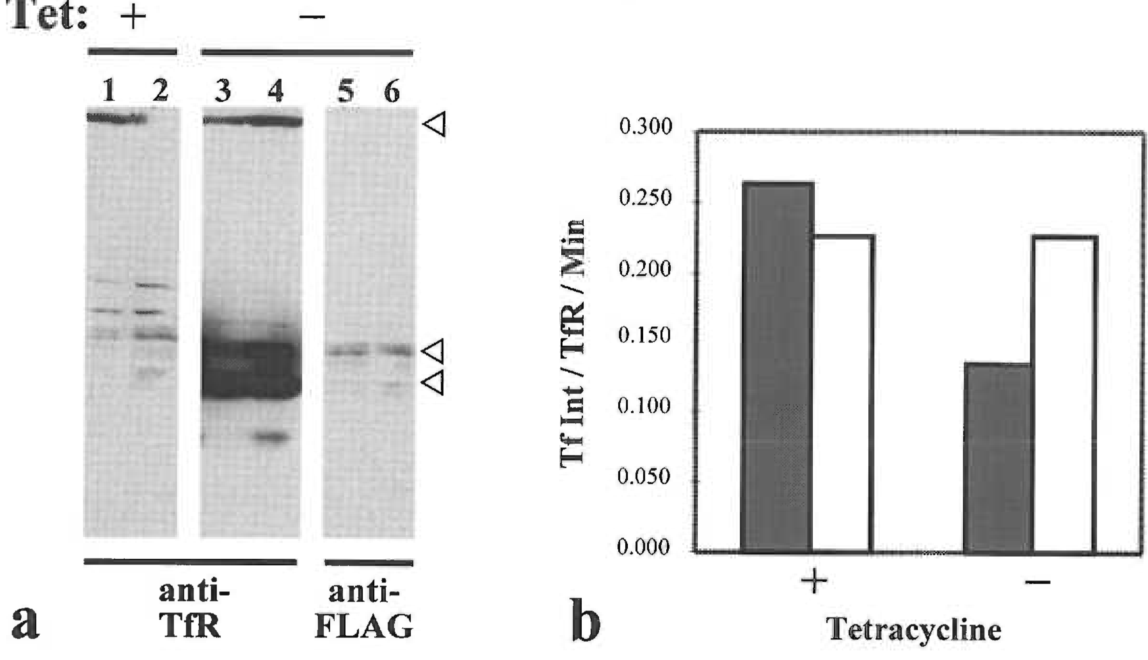


Figure 8. *Competition by Overexpression Requires an Intact Internalization Signal.* (a) Cell lysates from either the 150Flag (lanes 1, 3, 5) or 150AAFlag (lanes 2, 4, 6) cell lines, grown in the presence or absence of tetracycline, were run on SDS-polyacrylamide (12%) gels under reducing conditions. After western transfer to nitrocellulose, blots were probed with mouse monoclonal antibodies to either the TfR cytoplasmic domain or Flag epitope, and immunodetected with horseradish peroxidase-conjugated secondary antibodies. The middle arrow indicates the position of the 150Flag and 150AAFlag proteins. The lower arrow indicates the degradation products of each of these proteins

(note anti-FLAG detection). The upper arrow indicates the position of full length endogenous TfR. (b) Uptake assays were performed on both cell lines using 50 nM ^{125}I-Tf. For nonspecific uptake measurements, 12.5 μM unlabeled Tf was included in the incubation media. Rates for 150Flag (gray bars) and 150AAFlag (white bars) are shown. Generation of 150Flag and 150AAFlag cell lines, western blotting, and uptake experiments were performed by Frank Green.

CHAPTER 3

Structural Features of the Cytoplasmic Domain of the Transferrin Receptor

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Manuscript in preparation.

SUMMARY

Internalization of the transferrin receptor requires the presence of the amino acid motif YTRF in the cytoplasmic domain. In particular, the tyrosine has been shown to play a crucial role in the proper targeting of the TfR to clathrin-coated pits while on the plasma membrane. Here we describe the expression of the entire cytoplasmic domain of the TfR in *Escherichia coli* using the pET-11 expression vector system. This domain has been purified by a combination of gel filtration and ion exchange chromatography techniques. Analysis of the TfR cytoplasmic domain by circular dichroism reveals a flexible structure that possesses approximately 40% β-turn structure, in accord with speculations that β-turn structures play a significant role in endocytosis. Though recent evidence suggests a role for the μ_2 subunit of clathrin adaptor AP2 in the direct binding of receptor internalization signals, the TfR cytoplasmic domain does not associate with purified clathrin-coats *in vitro*.

INTRODUCTION

The transferrin receptor (TfR), a type II transmembrane glycoprotein, is important for the transport of iron into the cell (for review see (Enns et al., 1996)). Regardless of whether or not its ligand transferrin (Tf) is bound, the TfR undergoes repeated cycles of endocytosis via clathrin-coated pits, transport through the early endosome, and recycling back to the plasma membrane. Like many receptors undergoing clathrin-mediated endocytosis, sequestration to clathrin-coated pits requires the presence of a short sequence of amino acids in the cytoplasmic domain which define an internalization signal (for reviews see (Sandoval and Bakke, 1994; Trowbridge et al., 1993)).

The signal for internalization of the human TfR, is contained within the amino acid motif YTRF, which is found at position 20-23 in the cytoplasmic domain (Collawn et al., 1993). This sequence falls into the class of internalization signals generally referred to as the tyrosine-based motifs, which is defined by the presence of a crucial tyrosine in the context of a loosely defined sequence of 4-6 residues. This tyrosine was proposed to play a role in the formation of a specific secondary structure that is recognized by components of the coated pit. When tyrosine-based motifs from the TfR and the low-density lipoprotein receptor (LDLR) were compared against a database of protein crystallographic structures, a common theme among analogs to the two sequences, was that they favored tight-turn conformations (Collawn et al., 1990). Physical evidence to support these analytical studies was generated by independant labs that were performing NMR analysis on synthetic peptides made from the cytoplasmic domains of three

proteins known to contain tyrosine-based internalization signals: the LDLR, lysosomal acid phosphatase, and the insulin receptor (Backer et al., 1992; Bansal and Gierasch, 1991; Eberle et al., 1991). The results of the analysis was that each of these peptides formed a secondary structure that correlated with a type I β-turn.

In the present study, the cytoplasmic domain of the transferrin receptor, purified from a recombinant bacterial expression system, is used to perform a series of studies on the secondary structure and functional properties of this domain. Analysis of the purified cytoplasmic domain by circular dichroism demonstrates that this soluble portion of the TfR displays a high level of β-turn (nearly 40%), with very little other secondary structure. This is in accord with the theoretical observations about the structure information contained within the amino acid sequence of the protein (Collawn et al., 1990).

Following the concept that AP2 interacts directly with the cytoplasmic domains of internalizing receptors, a set of *in vitro* binding experiments were performed between the purified cytoplasmic domain and isolated clathrin coats. Even using crosslinking reagents to enhance potential transient interactions between the TfR cytoplasmic domain and clathrin coats, no binding is detected.

MATERIALS AND METHODS

Constructs and Mutagenesis—The cytoplasmic domain of the human transferrin receptor was amplified by PCR from the TfR/pZipNeoSVX (Williams and Enns, 1991) using the oligonucleotides NcoIBeg (5'-cag ttc aga acc atg gat caa gct aga-3'), which creates a Nco I site in place of the first methionine of the receptor (maintaining the second methionine), and BamZ62 (5'-agt ccc ata gcg gat cct tcc act tca-3'), which produces a stop site at position 62 followed by a Bam HI restriction site. The PCR product was purified using Promega's Wizard PCR Preps kit, and digested with restriction enzymes Nco I and Bam HI. The resulting fragment (Z62) was isolated by gel electrophoresis, and repurified with Promega's Wizard PCR Preps kit. pET-11d (Novagen), a bacterial expression vector under the control of the lac promoter, was digested with Nco I and Bam HI, treated with calf intestinal alkaline phosphatase (Boehringer Mannheim), and gel purified as described above. The PCR fragment and pET-11d vector were ligated, resulting in the final construct Z62/pET-11d.

Large scale production and purification of the cytoplasmic domain—For large scale production, the BL21(DE3) strain of *Escherichia coli* transformed with the Z62/pET-11d plasmid, was inoculated into a 500 ml culture of ZBAmp broth (10 g/l tryptone (Gibco), 5 g/l NaCl (Sigma), 25 μg/ml ampicillin (Sigma)). The culture was grown overnight shaking at 37°C, and used to inoculate six liters of ZBAmp broth in six Fernbach flasks the following morning. The cultures were then grown under the same conditions until an OD_{600} of ~0.6 was reached, at which point induction was initiated by adding IPTG to

each flask to a final concentration of 0.2 μg/ml. After 2.5 to 3.0 more hours of shaking at 37°C, the culture was centrifuged at 30,000 x g 20 min. and resuspended in 10 ml phosphate buffer (0.02 M KH_2PO_4, pH 7.4, 1 μg/ml leupeptin, 1 mM EDTA). The bacteria were lysed by French press, the debris cleared by centrifugation at 30,000 × g for 20 min. The extract was heat-treated at 70° C 10 min., and centrifuged at 30,000 × g for 20 min. The cleared lysate was then passed over a ~500 ml G-50 Sephadex column (2.5 cm x 100 cm) at 30 ml/hr, collecting 60 x 5 ml fractions. The fractions, monitored by OD_{280} were also examined by SDS-PAGE (Laemmli, 1970) and Coomassie blue staining. Those fractions containing the cytoplasmic domain (usually around 38-42) were then passed over a 4 ml DEAE-Sephacel column by gravity flow, in phosphate buffer with a linear NaCl gradient of 0 - 0.3 M NaCl. Ten fractions were collected, and examined by spectrophotometry at $OD_{260/280}$ and SDS-PAGE Coomassie blue. Those fractions (3-5) containing the cytoplasmic domain were pooled and concentrated with Centricon concentrators (3,000 MW cutoff) to a final concentration of 0.5 - 1 mg/ml in 100 μl, for circular dichroism, amino acid analysis, sequencing and cell injections. The $OD_{260/280}$ was ~ 1.2. Low molecular weight markers (Sigma MW-SDS-17S) included myoglobin (17 kDa), myoglobin I + II (14.4 kDa), myoglobin I + III (10.6 kDa), myoglobin I (8.2 kDa), myoglobin II (6.2 kDa), glucagon (3.4 kDa) and myoglobin III (2.5 kDa).

Circular dichroism analysis—Purified TfR cytoplasmic domain in 50 mM KH_2PO_4 buffer at pH 6.5 was analyzed by circular dichroism. Spectra from 180 to 260 nm were recorded on a Jasco J-500A instrument, and the results interpreted using the variable selection method (Compton and Johnson, 1986; Compton et al., 1987; Manavalan and

Johnson, 1987). The concentration of the samples was determined by amino acid analysis.

Microinjections—Microinjection apparatus consisted of an Eppendorf Micromanipulator 5171 and Microinjector 5242, attached to a Zeiss IM35 inverted fluorescence microscope. Injection pressure (setting P2) of 70-200 hPa and duration of 0.5 to 1.0 seconds resulted in a ~5-10% increase in total cell volume. Injection buffer (132 mM K·Aspartate, 1 mM $CaCl_2$, 2 mM EGTA, 2 μM MgATP, 5 mM HEPES, 64 mM Mannitol, pH 7.0) was used in buffer alone injections, as well as injections of purified TfR cytoplasmic domain (@ 100 ng/μl). All injections included the coinjection marker, lysine-fixable tetramethylrhodamine dextran 10,000 MW (@ 10 μg/ml), which was used for later identification of injected cells.

In preparation for microinjection experiments, A431 cells were plated at low density onto sterile coverslips in 6-well plates, and grown for at least 2 days at 37°C and 5% CO_2 to assure strong attachment. Coverslips were transferred individually to a 6 cm plate containing fresh growth media for each series of microinjections. Cells were allowed to "recover" for 10 minutes at 37°C and 5% CO_2 before adding FITC-transferrin to a final concentration of 10 μg/ml. Following incubation for an additional 10 minutes at 37°C and 5% CO_2 to ensure uptake of FITC-transferrin into endosomes, cells were fixed in 3% paraformaldehyde, and mounted onto slides for observation.

Assay for Coat Interactions with TfR Cytoplasmic Domain—2 μg of purified TfR cytoplasmic domain was combined with 2.7 μg of purified clathrin-coats (courtesy of Dr.

Jim Keen, Thomas Jefferson University) in buffer A (5 mM Hepes, 100 mM sodium potassium tartarate, 0.5 mM EDTA, 0.25 mM $MgCl_2$, pH 7.2). After incubation on ice for 30 minutes, the cleavable crosslinking reagent DTSSP (3,3'-dithiobis(sulfosuccinimydl propionate) - Pierce) was added to a final concentration of 0.1 mM, and allowed to react for an additional 30 minutes on ice. The entire sample was loaded onto a 10-30% sucrose gradient in 2 ml ultracentrifuge tubes (Beckman) and spun at 4°C in a Beckman TLS-55 rotor at 22000 RPM for 5 hours. Ten to twelve 200 μl fractions were collected, and run on a reducing 8-18% gradient tricine buffered SDS-polyacrylamide gel (Schagger and von Jagow, 1987). For comparison, samples were also spun at 75,000 RPM in a Beckman TLA 100.1 rotor to separate out only the soluble and insoluble fractions.

After transfer to nitrocellulose, the resulting western blot was cut into two pieces: an upper piece representing proteins with a MW higher than 43 kDa, and a lower piece representing proteins less than 43 kDa. The top portion was probed with a 1:1000 dilution of mouse monoclonal antibody to α-adaptin (clone 100/2 from Sigma) and the bottom portion with a 1:10,000 dilution of mouse monoclonal antibody to the TfR cytoplasmic domain (H68.4 from Zymed). Both portions were probed with a 1:10,000 dilution of horseradish peroxidase-coupled goat anti-mouse secondary antibody (TAGO) and detected with chemiluminesence reagent (Pierce). High molecular weight markers (Sigma SDS-6H) included myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa). Low molecular weight markers (BRL 26000-018) included ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), lysozyme

(14.3 kDa), bovine trypsin inhibitor (6.2 kDa), insulin β-chain (3.4 kDa), and insulin α-chain (2.3 kDa).

RESULTS

Expression of the TfR Cytoplasmic Domain in E. Coli. The human transferrin receptor was mutated to create a stop codon at position 62 in place of the normal cysteine residue, and then introduced into the pET-11d vector for expression in *Escherichia coli* BL21(DE3). The pET-11d vector contains a Nco I cloning site with an ATG translation start site, under the control of the lac promoter. Induction of the bacteria with IPTG results in a high level of expression of the cloned protein. Comparison of cell extracts of the induced bacteria with those of uninduced bacteria revealed the presence of a significantly enhanced band at ~6.8 kDa, the expected size of the transferrin receptor cytoplasmic domain (Fig. 1A - Lane I). Heat treatment of bacterial extract at 70°C for 10 minutes precipitated a large number of nonspecific proteins which could then be removed by centrifugation, without significantly affecting the yield of the expressed product (Fig. 1A - Lane U). Heat denaturation was later removed from the purification scheme due to concerns about the effect on the structure and function of the TfR cytoplasmic domain.

The ~6.8 kDa band was recognized by an antipeptide antibody (Yoshimori et al., 1988) to the cytoplasmic domain (aa8-27) of the transferrin receptor (Fig. 1B). To confirm that the observed band was that of the TfR cytoplasmic domain, the band was subjected to protein sequencing (performed by Barb Root at Bristol-Meyers, Syracuse, N.Y.). The first six amino acids of the protein corresponded to the first six amino acids of the transferrin receptor, although the N-termini were heterogeneous beginning with methionine (the first encoded amino acid) and aspartic acid (the second coded amino acid).

This was probably the result of partial hydrolysis of the N-terminal methionine-aspartic acid bond.

Purification of the TfR Cytoplasmic Domain. The putative cytoplasmic domain was isolated initially by gel filtration chromatography on a ~500 ml Sephadex G-50 sizing column. The profile of the eluted proteins is shown in Fig. 2. Fraction 26 generally contains the bulk of the eluted proteins, but a small peak at fractions 38-42 signifies the presence of the receptor cytoplasmic domain. Although the protein appeared pure with respect to Coomassie blue staining, the $OD_{260/280}$ ratio was ~2.0, suggesting the presence of nucleic acid in a concentration too high for successful circular dichroism analysis. The protein was further purified by anion exchange chromatography using a 4 ml DEAE-Sephacel column and a linear NaCl gradient (0 to 300 mM). The purified protein, which usually comes off in fractions 3-5, had an $OD_{260/280}$ ratio of 1.0 to 1.4, closer to the predicted value of 0.9 for a peptide with a tyrosine/phenylalanine composition of the transferrin cytoplasmic domain. The protein was concentrated in 2 ml spin columns (Centricon) to a final concentration of 0.5 to 1.0 mg/ml.

Secondary Structure of the TfR Cytoplasmic Domain is Predominantly β-turn. The presence of secondary structure in the cytoplasmic domain was determined by subjecting the purified and concentrated protein to circular dichroism analysis (Fig. 3), and interpreting the data using the variable selection method (Compton and Johnson, 1986; Compton et al., 1987; Manavalan and Johnson, 1987). The results are consistent with the presence of 38±4% β-turn structure, and very little additional structure (12±5% α-

helix, 13±8% anti-parallel β sheet and 0±1% parallel β sheet). Garnier analysis of the amino acid sequence predicts 20% β-turn, and that the most likely areas for β-turn structure reside in amino acids 20-23, the location of the YTRF internalization signal, and amino acids 30-33 (Garnier et al., 1978).

Assay for function of the purified TfR cytoplasmic domain by microinjection into live cells. The structure of the TfR cytoplasmic domain was determined using the product of bacterial expression and column purification procedures. To show that the resulting purified protein was functionally representative of the cytoplasmic domain of the full length TfR, a microinjection assay was developed. The assay would involve microinjecting a human cell line that expresses an endogenous level of wild type TfR with the purified cytoplasmic domain protein, and analyzing the efficiency of endocytosis of a fluorescently-tagged transferrin. The hypothesis was that the purified cytoplasmic domain protein, containing the signal for internalization of the TfR, would be able to interfere with components of the endocytic machinery responsible for the recognition of the TfR. Interferance would result in a decreased internalization efficiency of fluorescently-tagged transferrin, and would be specific to the presence of the purified TfR cytoplasmic domain protein.

Human A431 cells were chosen for microinjection, since they endogenously express wild type TfR, and are relatively easy to inject due to their size. A431 cells were microinjected with either a control buffer, or buffer containing 100 ng/μl of purified TfR cytoplasmic domain protein. Using tetramethylrhodamine-dextran (Rh-Dextran) to

identify injected cells (Fig. 4 - left panels), comparisons were made to determine if the various injections had any effect on internalization efficiency.

Normal internalization of FITC-transferrin appears as puncate structures visible in the cytoplasm of the cell (see uninjected cells of upper right-hand panel of Fig. 4). This pattern of fluorescence is typical for the early stages of endocytosed FITC-Tf that is residing in the early endosome. If A431 cells are injected with a control buffer, the punctate structures are not affected (Fig. 4 - upper panels). However, injection of the purified TfR cytoplasmic domain (Z62) seems to have a variable effect (Fig. 4 - lower panels). Cells that received injections into the cytoplasm (cells marked with a 'c') show a loss of punctate staining that is replaced with a very diffuse pattern, reminiscent of surface-only fluorescence. This might indicate that endocytosis for the TfR is much reduced, and the FITC-transferrin is not being delivered to an internal compartment. Nuclear injections (cells marked with an 'n') retain the punctate pattern implicative of normal TfR endocytosis, suggesting that the purified protein cannot escape the nucleus to exert a competitive effect. If two similarly injected cells (marked with asterisks in the lower left panel of Fig. 4) are compared, it appears that one shows a reduced endocytosis phenotype, and one does not. Though the results of the assay were promising, they were not very repeatable. Successive attempts at duplicating these results were unsuccessful. Contributing factors might have come from the variablity of microinjection needles, differential cell plating, or possibly even stability of the Z62 protein. Regardless, the results from the assay were inconclusive.

The TfR cytoplasmic domain does not interact with clathrin-coats in vitro. Interaction of receptor cytoplasmic domains with clathrin adaptor proteins has long been thought to describe the mechanism by which receptors are sequestered into clathrin-coated pits. AP2 has been demonstrated to bind the cytoplasmic domains of a number of different plasma membrane receptors (Beltzer, 1991; Glickman et al., 1989; Pearse, 1988; Sosa et al., 1993), but never with a high enough affinity to account for recruitment of AP2 to the plasma membrane. The TfR cytoplasmic domain has never been convincingly shown to interact with AP2, perhaps because the affinity of association is too low to detect by conventional binding techniques. To improve the chances of detecting an interaction, a couple of strategies were employed. First, AP2 was used in the context of reassembled clathrin coats, made from purified AP2 and clathrin. The idea is that perhaps the clathrin adaptor can be presented in a more realistic manner, and show a higher overall affinity for receptor cytoplasmic domains. Second, purified TfR cytoplasmic domain was used in a relatively high concentration to drive association towards the putative bound product, allowing the possibility of detection despite a lower affinity. Finally, to enhance any potential low affinity protein:protein associations, a cleavable crosslinking reagent was used. The resulting assay involved combining 2 μg of purified TfR cytoplasmic domain and 2.7 μg of reassembled clathrin coats together in a test tube with a crosslinker, incubating on ice for 30 minutes, and running through a 10-30% sucrose gradient to separate individual components. If indeed the TfR cytoplasmic domain and AP2 were to interact under these conditions, then it was expected that the crosslinked product would sediment at about the same location in the sucrose gradient as the reassembled clathrin coats.

Before the experiment could be performed, it was necessary to determine if a positive result would be detectable with the amounts of each component used in the assay. The lower limit of detection for the mouse monoclonal antibody to the TfR cytoplasmic domain was about 1 ng (data not shown). To calculate the expected amount of TfR cytoplasmic domain that would interact with the clathrin coats, a few assumptions were made. The first assumption is that the TfR cytoplasmic domain (Z62) would bind to AP2 with first order binding kinetics. Thus, we could describe the binding with an equilibrium dissociation constant,

$$K_d = \frac{[A][B]}{[AB]}$$

where [A] and [B] are defined as the "free" forms of each component, and [AB] is the "bound" form. The total amount of each component used is then equal to the sum of the "free" and "bound" forms, or:

$$[\text{total } A] = [A] + [AB]$$
$$[\text{total } B] = [B] + [AB]$$

The concentrations for either Z62 or AP2 are derived from the amount of each component used, the MW of Z62 (6,832 Da) and AP2 (260,000 Da), and the assumption that the reassembled coats consist of 70% clathrin by weight, as shown:

$$\frac{2\ \mu\text{g Z62}}{200\ \mu\text{l}} \times \frac{\mu mole}{6832\ \mu\text{g}} \times \frac{10^6\ \mu\text{l}}{liter} = 1.46\ \mu M \text{ total Z62}$$

$$\frac{2.7\ \mu\text{g coats}}{200\ \mu\text{l}} \times \frac{300\ ng\ \text{AP2}}{1\ \mu\text{g coats}} \times \frac{nmole}{260{,}000\ ng} \times \frac{10^6\ \mu\text{l}}{liter} = 15.6\ nM \text{ total AP2}$$

The amount of "free" can be determined,

$$\text{free Z62} = [A] = (1.46\ \mu M \text{ total Z62}) - [AB]$$
$$\text{free AP2} = [B] = (15.6\ nM \text{ total AP2}) - [AB]$$

and used to substitute into the equation for K_d:

$$K_d = \frac{((1.46 \times 10^{-6}\ M) - [AB])((15.6 \times 10^{-9}\ M) - [AB])}{[AB]}$$

If we assume that the K_d =1 μM, taken from the low affinity AP2:receptor cytoplasmic domain binding results published previously, then solving for the concentration of total "bound" product, or [AB], we get a value of 9.5 nM. From this, we can determine the total amount of Z62 that would be expected to bind the original amount of clathrin coats:

$$\frac{9.5\ nmole}{liter} \times \frac{6832\ ng}{nmole} \times \frac{200\ \mu\text{l}}{1} \times \frac{liter}{10^6\ \mu\text{l}} = 13\ ng \text{ bound Z62}$$

Thus, using 2 μg of Z62, and 2.7 μg of AP2 in a 200 μl reaction, and assuming first order kinetics with a K_d of 1 μM, we can expect to see ~13 ng of Z62 cosedimenting with the reassembled clathrin coats, well within the ranges of detection of the mouse monoclonal TfR antibody.

The results of the assay are shown in Figure 5. The composition and purity of reassembled clathrin coats, generously donated by Dr. Jim Keen of Thomas Jefferson Univeristy, were verified by running on a reducing SDS-polyacrylamide gel, and detecting with Coomassie blue staining. Z62 and clathrin coats were combined into 200 μl of buffer and reacted with the crosslinking reagent DTSSP for 30 minutes on ice. After the crosslinked sample was layered onto a 10-30% sucrose gradient, and spun at 40,000 × g for 5 hours at 4°C, the resulting sample was collected into 10-12 fractions. Each fraction

was run on an 8-18% tricine-buffered gradient SDS-polyacrylamide gel, and transferred to nitrocellulose. The resulting western blot (Fig. 5a) was divided horizontally across the 43 kDa molecular weight marker, and the upper and lower portions probed separately to detect the position of α-adaptin at ~100 kDa (upper portion) or the TfR cytoplasmic domain at ~6.8 kDa (lower portion), respectively.

Nearly all of the Z62 protein is found in the first few fractions, and α-adaptin runs in fractions 6 thru 9. No Z62 protein is detected in the same fractions as α-adaptin. The lack of cosedimentation, when taken with the hypothesis described above, would imply that there is no detectable interaction between the Z62 protein and AP2 in our binding assay. However, both the Z62 protein and AP2 are found as insoluble aggregates in the pellet of the sucrose gradient. To determine if the presence of Z62 in the pellet is due to an AP2 association, the assay was repeated in the presence or absence of clathrin coats, and spun at 75,000 RPM in a Beckman TLA 100.1 rotor to separate the insoluble fraction. No significant increase in the amount of Z62 in the pellet is detected in the presence of clathrin coats, when compared to Z62 alone (Fig. 5b - see lower arrow), nor is there an observable change in the soluble fraction of Z62. This implies that the fraction of Z62 found in the pellet is not specific to the presence of clathrin coats. Also present in the pellet is a ladder of bands detectable with the TfR antibody that most likely represents multimers of the crosslinked Z62 protein (Fig. 5b - asterisks).

DISCUSSION

The TfR internalization signal YTRF has been shown to be necessary for the efficient clustering of this receptor into clathrin-coated pits (Collawn et al., 1993). The mechanism of recognition by the endocytic mechanism remains to be determined, but probably involves the interaction of coated-pit proteins, either with the internalization signal directly, or with secondary connector proteins.

To examine the structural features and functional properties of the TfR internalization signal, we were interested in expressing and purifing the TfR cytoplasmic domain as a soluble protein. Protein expression was accomplished using the pET-11 bacterial expression system. The purified protein runs at ~6.8 kDa, which is consistent with the prediction based on a peptide length of 61 amino acids.

The purified TfR cytoplasmic domain was analyzed by circular dichroism to determine if there were any prominant structural features. Though it was found to be a flexible protein, possessing very little structure (α-helix, β-sheet, etc.), one characteristic of the TfR cytoplasmic domain that did come out of the analysis, was the tendancy for the formation of β-turn in secondary structure (nearly 40%). This result supports earlier predictions that the region immediately surrounding the tyrosine at position 20, which is critical for the endocytosis of the TfR, conforms to a β-turn, a feature proposed to be necessary for the proper targeting to clathrin-coated pits (Collawn et al., 1990).

Attempts to demonstrate the functionality of the purified TfR cytoplasmic domain have proven unsuccessful. One method employed was to examine the competitive properties of the purified protein when microinjected into the cytoplasm of

living cells. The idea was to demonstrate a reduced efficiency of FITC-transferrin endocytosis in cells injected with the TfR cytoplasmic domain. Although the effect was seen a couple of times, results from these experiments were, for the most part, ambiguous and inconsistant.

The μ_2 subunit of the clathrin adaptor complex AP2 has been proposed to play an important role in the recognition and recruitment of proteins bearing internalization signals (Boll et al., 1996; Ohno et al., 1996; Ohno et al., 1995; Rapoport et al., 1997). Here, we employ several strategies to develop a more efficient binding assay in the hopes of increasing the probability of detection of a low affinity association. Calculations based on a low affinity dissociation constant of 1 μM were used to determine that detection of bound TfR cytoplasmic domain was a feasible venture. Nevertheless, we present evidence that the TfR cytoplasmic domain protein does not associate with purified clathrin-coats *in vitro*, even in the presence of a crosslinking reagent.

The inability of the TfR cytoplasmic domain to crosslink to purified clathrin coats suggests a number of possibilities: 1) the component of the endocytic machinery necessary for cytoplasmic domain interaction is not present in purified clathrin coats, 2) the purified cytoplasmic domain cannot form the structure that is necessary for an *in vitro* interaction with clathrin coats, possibly requiring membrane attachment for proper conformation, or 3) components of the clathrin coat that are necessary for interaction with the cytoplasmic domain are inaccessible.

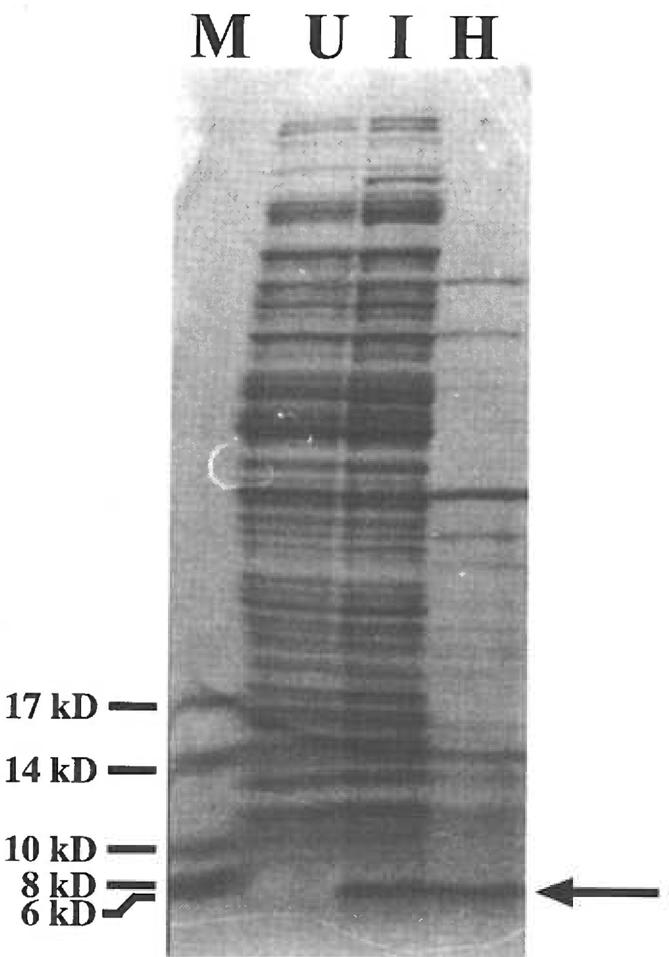

Figure 1A. *Expression of the cytoplasmic domain of the transferrin receptor in E. coli.* Whole cell lysates prepared from IPTG induced (I) or uninduced (U) BL21(DE3) *E.* coli, transformed with a bacterial expression plasmid containing the sequence for the cytoplasmic domain of the transferrin receptor (Z62/pET-11d), were run on an 10-20% tricine-buffered polyacrylamide gradient gel, and detected with Coomassie blue staining. A predominant band at ~6.8 kDa representing the transferrin receptor cytoplasmic domain (see arrow), is detectable only in the lane containing extract from IPTG induced cells. Lane H indicates whole cell lysates prepared from IPTG induced cells that were heat treated for 10 minutes at 70°C. Work shown was performed by Tony Williams, Ph.D.

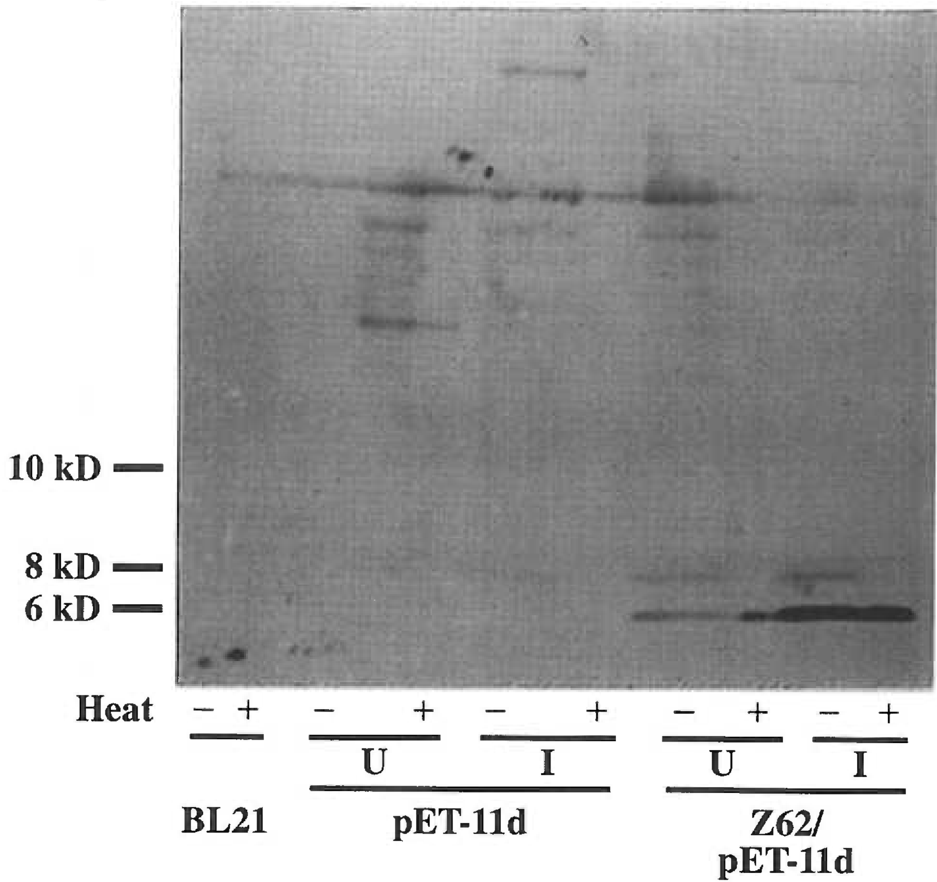

Figure 1B. *Immunodetection of the transferrin receptor cytoplasmic domain.* Heat treated and non-heat treated extracts from BL21(DE3) *E. coli* either alone, or transformed with pET-11d or Z62/pET-11d, were separated by gel electrophoresis, transferred to nitrocellulose, and detected with an antibody to the cytoplasmic domain of the transferrin receptor (Yoshimori et al., 1988). The identity of the ~6.8 kDa protein was further verified by protein sequence analysis. Work performed by Tony Williams, Ph.D.

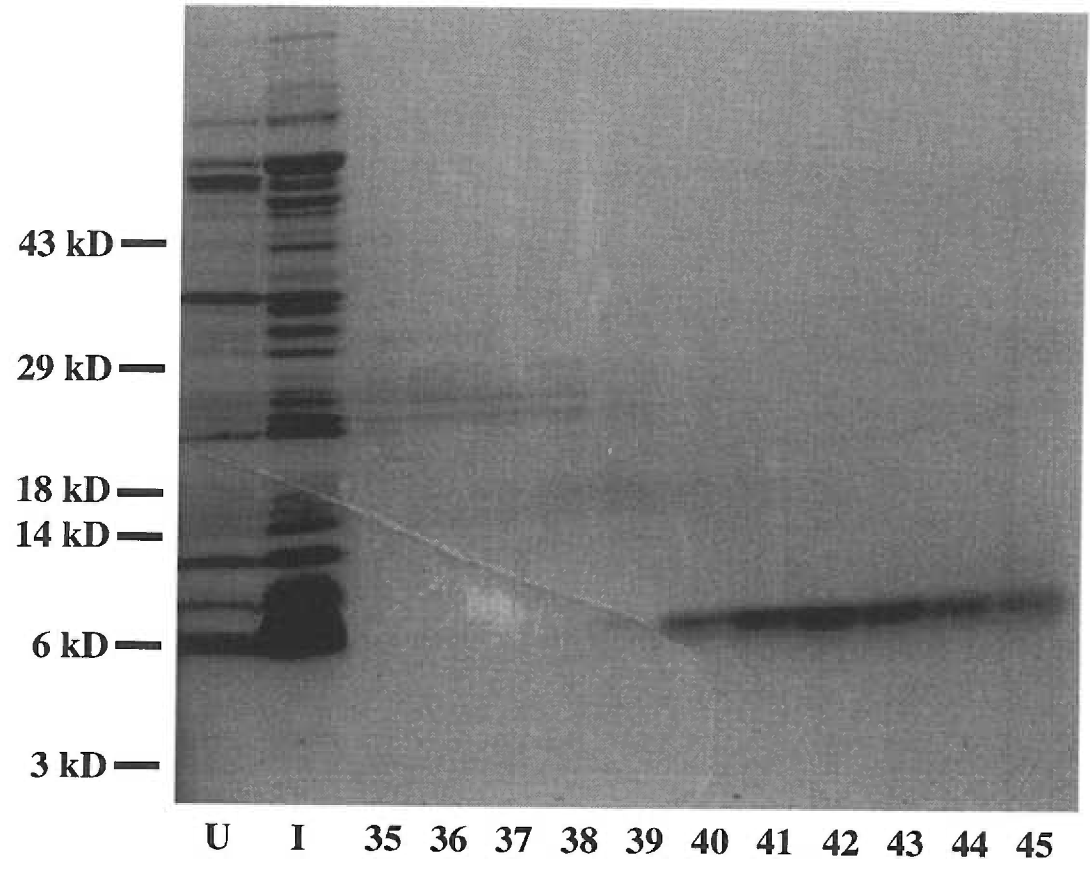

Figure 2. *Gel filtration of the transferrin receptor cytoplasmic domain on Sephadex G-50.* Heat-treated extracts of Z62/pET-11d transformed BL21 cells were separated by Sephadex G50 column chromatography. Fifty fractions of 5 ml each were collected, and samples from each fraction were analyzed by SDS-PAGE and Coomassie blue staining. Whole cell extracts from uninduced (U) and IPTG induced (I) were loaded alongside column fractions 35-45, which contain partially purified transferrin cytoplasmic domain. Work performed by Tony Williams, Ph.D.

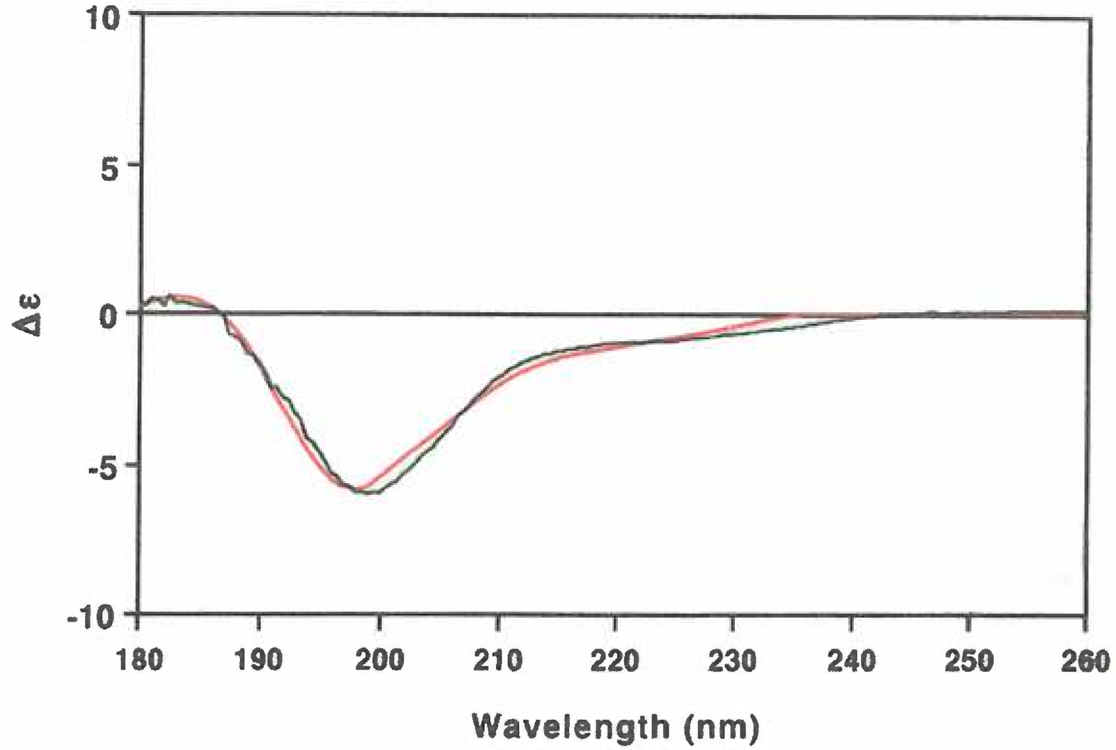

Figure 3. *Circular dichroism (CD) spectral analysis of the cytoplasmic domain.* The purified transferrin receptor cytoplasmic domain was concentrated to a concentration of 0.5 to 1.0 mg/ml, and analyzed by circular dichroism at wavelengths 180 to 260 nm. The green line shows the smoothed CD data analysis of the transferrin receptor cytoplasmic domain. The red line represents the best curve fit according to specific secondary structural analysis. Results of the circular dichroism analysis were as follows: 12±5% α-helix, 13±8% anti-parallel β sheet, 0±1% parallel β sheet, and 38±4% β-turn structure. Garnier analysis predicted the presence of 20% β-turn, and favored the amino acid regions from 20-23 and 30-33 for the formation of a β-turn. Circular dichroism and analysis performed by Hans Peter Bachinger, Ph.D.

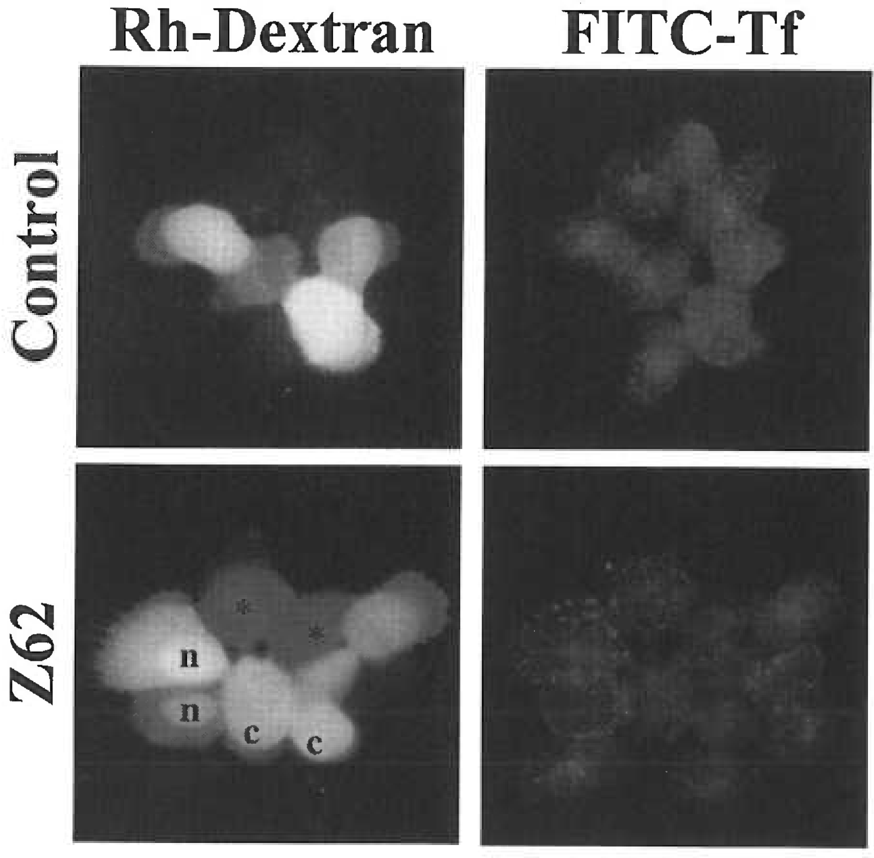

Figure 4. *Microinjection of the TfR cytoplasmic domain into A431 cells.* A431 cells on coverslips were microinjected with either buffer alone (top panels) or buffer containing TfR cytoplasmic domain at 100 ng/µl (bottom panels), along with a coinjection marker (tetramethylrhodamine-dextran 10,000 MW, Molecular Probes). Cells were incubated with 10 µg/ml FITC-transferrin conjugate (Molecular Probes) at 37°C and 5% CO2 for 10 minutes prior to fixing and mounting. Injected cells were identified by rhodamine-fluorescence (left panels) and uptake of FITC-transferrin was detected as punctate staining visible in the cytoplasm (right panels). n, denotes nuclear injection; c, denotes cytoplasmic injection. Photographs are at 400× magnification.

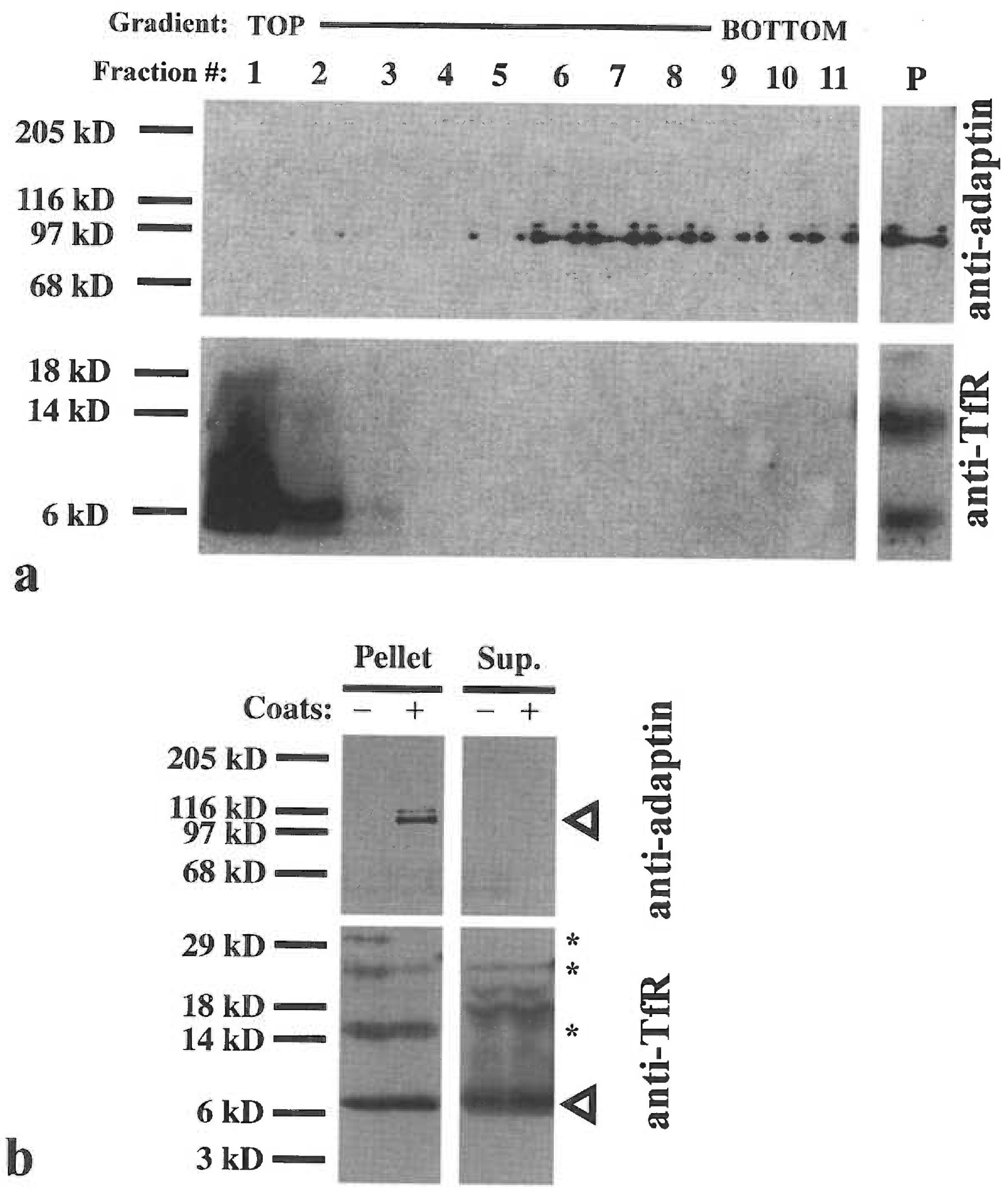

Figure 5. *Lack of interaction between purified TfR cytoplasmic domain, and purified coats in vitro.* (a) Purified TfR cytoplasmic domain and clathrin coats were incubated in the presence of the crosslinker DTSSP (0.1 mM) for 30 minutes on ice. Following separation on a 10-30% sucrose gradient, fractions were loaded and run on a reducing 8-

18% tricine-buffered gradient SDS-polyacrylamide gel and transferred to nitrocellulose. The resulting western was divided into two pieces and probed with either a mouse monoclonal antibody to α-adaptin (upper portion) or a mouse monoclonal antibody to the cytoplasmic domain of the TfR (lower portion). Both portions of the blot were detected with a horseradish peroxidase-coupled anti-mouse secondary antibody. (b) Purified TfR cytoplasmic domain and clathrin coats were incubated in the presence of the crosslinker DTSSP (0.1 mM) for 30 minutes on ice, spun at 75000 RPM in a Beckman TLA 100.1 rotor, and pellet/supernatants run on a reducing 8-18% tricine-buffered gradient SDS-polyacrylamide gel. After transfer to nitrocellulose, westerns were probed as described above for part (a). Arrows indicate location of either adaptin (upper) or TfR cytoplasmic domain protein (lower). Asterisks denote multimers of TfR cytoplasmic domain protein that are typically seen in the pellet.

SUMMARY AND CONCLUSIONS

The structure of the clathrin coat on the cytoplasmic face of the membrane provides a basis for the hypothesis that the AP2 complex, which is responsible for the assembly and recruitment of clathrin, may play a primary role in the recognition of either dileucine-based or tyrosine-based receptor internalization signals. This idea has gained much support in the last several years, stemming from the original data that adaptor complexes could interact weakly with the cytoplasmic domains of receptors immobilized to agarose (Glickman et al., 1989; Pearse, 1988). The yeast two-hybrid identification of the AP2 μ_2 subunit as the internalization signal recognition protein has stirred extensive research into the properties of this interaction (Ohno et al., 1995). *In vitro* binding assays have further defined these interactions as specific to the tyrosine and dependent on clathrin and/or phosphoinositides for efficient binding (Rapoport et al., 1997). Additionally, evidence from surface plasmon resonance measurements may support the role of the AP2 μ_2 subunit in the recognition of the dileucine motif (Heilker et al., 1996).

The suggestion that plasma membrane receptor cytoplasmic domains provide the necessary site for recruitment of clathrin adaptors remains a controversial issue. Evidence in favor of this hypothesis comes from work by independant labs that show an increase in either clathrin lattice (Miller et al., 1991) or clathrin-coated pits (Iacopetta et al., 1988) when the human transferrin receptor is overexpressed. Similarly, overexpression of the CD-M6PR showed a correspondant increase in the amount of AP1 recruited to the Golgi membrane (Le Borgne and Hoflack, 1997). In contrast, work by Santini and coworkers using an immobilized antigen for IgE Fc receptors, demonstrated that although they could

aggregate a large number of activated receptors at the plasma membrane, no obvious increase in the amount of recruited AP2 could be detected (Santini and Keen, 1996). Work from our lab, as presented in Chapter 1, demonstrates that TfR overexpression has no effect on the amount of plasma membrane associated AP2 (Warren et al., 1997).

Although recent publications suggest that AP2 is directly responsible for the recognition of all tyrosine-based and dileucine-based internalization signals, some amount of controversy surrounds this idea. Wiley has shown that endocytosis of the EGFR in A431 cells can be saturated, without having an effect on the internalization of the TfR (Wiley, 1988). This would suggest that the TfR is utilizing a mechanism that is not affected when EGFRs have saturated some component of the endocytic apparatus. Furthermore, though there is evidence for a specific AP2 binding site on the EGFR cytoplasmic domain most likely centered around a tyrosine-based internalization signal (Nesterov et al., 1995; Nesterov et al., 1995; Sorkin et al., 1996; Sorkin et al., 1995), the interaction does not appear to be necessary for the efficient endocytosis of this receptor (Nesterov et al., 1995).

The results presented here argue for a modification of the current model for the recognition of internalization signals by the endocytic machinery. In chapter 1, I demonstrated that the TfR could be overexpressed to the extent of saturation of endocytosis for this receptor, but had no adverse effect on the rate of internalization of the EGFR. This was not too surprising, since it corroborated the results from Wiley's previous studies of the EGFR in A431 cells. However, these results are at odds with earlier work by Rothenberger and coworkers who demonstrated that overexpression of TfR in mouse cells had no significant effect on the rate of uptake per receptor when

compared to cells expressing lower amounts of TfR (Rothenberger et al., 1987). They were only able to detect a saturable event at high receptor numbers when they examined the rate of iron uptake in the same cells. The authors explanation suggested that the decreased efficiency of iron uptake was due to slowed recycling, a result of saturating the rate of exocytosis, rather than endocytosis. The primary difference with this data from our results is the method used to measure the rate of TfR internalization. Rothenberger and coworkers prebound ^{125}I-Tf at 4°C before measuring uptake rates at 37°C for various timepoints. Our assay involved adding saturating amounts of ^{125}I-Tf at 37°C and immediately measuring uptake. Since endocytosis is normally blocked at 4°C, the rates of TfR internalization measured by Rothenberger and coworkers might not reflect steady state transferrin uptake, but rather an artifact of endocytosis recovery.

In chapter 2, the overexpression of TfR, LDLR, and EGFR was examined, as it related to the endocytosis of these receptors and other surface proteins. Though each of these receptors was capable of saturating the rate of their own internalization, there was no competition between different receptors. These results were surprising as other groups had previously postulated that all tyrosine based motif internalization signals would interact with AP2. The implication is that the mechanisms for endocytosis of each of these receptors varies to the extent that they are independant of each other. The Lamp-1 protein, which is present in small amounts on the plasma membrane, and contains a functional tyrosine-based internalization signal, appears to be affected by overexpression of the TfR, and is shown to increase by about 45% of normal in total surface number. This increase, though small, is significant and may account for a reduced ability of Lamp-1 to enter coated pits and be internalized. That the TfR endocytic signal

may compete with the signal from Lamp-1 supports the concept that at least some of the components of the recognition machinery are shared. Marks and colleagues have shown that proteins exhibiting a YXX∅ internalization motif in their cytoplasmic domain utilize a common pool of saturable components for efficient endocytosis (Marks et al., 1996).

The evidence presented herein support a model for multiple distinct endocytic recognition mechanisms. At least one of these mechanisms may involve the direct interaction of receptor tails with the clathrin adaptor proteins. Alternatively, intermediate proteins may be responsible for recognizing a given internalization signal, and delivering the targeted protein to the coated pit. A role similar to this has been attributed to the EGFR substrate, Eps15 (Benmerah et al., 1996; van Delft et al., 1997). Eps15, which is phosphorylated by the EGFR after its activation with EGF, shows a strong association with the receptor as well as with α-adaptin and clathrin. The necessity of these interactions for the proper endocytosis of activated EGFR have not been established, but suggest an alternate model for EGFR recruitment to coated pits. The interaction seen previously between the EGFR and AP2 may in fact be a secondary event, possibily necessary for the continued association of the receptor with the coated pit.

The identification of these intermediate proteins, or sorting connectors, are the first step to better understanding the events that occur during the recognition of different internalization signals, and rapid clustering into clathrin coated pits. Two-hybrid library screening has identified interactions between the μ_2 subunit and tyrosine-based internalization signals, but has failed to reveal other proteins that may be involved (Ohno et al., 1995). Perhaps the environment of the yeast 2-hybrid screen is suitable for

identifying the μ_2:signal interaction but discourages an association with the theoretical sorting connectors. The method employed by Ohno et. al. was to increase the probability, or strength of the association *in vitro* by including a triple repeat of the TGN38 internalization signal sequence. It is possible that this technique artificially enhanced an otherwise insignificant association, despite the demonstration that the interaction was specific, and required an intact internalization signal (Ohno et al., 1995). Perhaps individual receptor internalization signals could interact with the same protein(s), but to different binding domains. This seems reasonable, except that it does not provide a good explanation as to why the EGFR can saturate its own internalization rate at a receptor density that is much lower than that of either the TfR or the LDLR.

Though other groups have demonstrated that the YTRF motif of the TfR can associate with the μ_2 subunit of AP2 when it is tagged to the end of a 26 amino acid sequence from TGN38, an interaction within the actual context of the TfR has not been shown. Attempts at utilizing crosslinking reagents to reveal interactions between a purified form of the entire TfR cytoplasmic domain and clathrin coats *in vitro* were unsuccessful but could be due to a number of problems, as detailed in the discussion of Chapter 3.

Identification of the component necessary for the rapid and efficient internalization of the TfR, will require some different approaches than have been tried in the past. In order for a yeast 2-hybrid library screen to prove useful, I believe a couple of problems will have to be addressed. The first of these problems is in the creation of a TfR cytoplasmic domain "bait" construct. Every 2-hybrid vector system that is currently available functions by generating the "bait" by fusing the target protein to the

C-terminus of a transcriptional activator DNA-binding domain. Since the TfR is a type-II transmembrane protein, this means that the normally free N-terminal end of the cytoplasmic domain, would now be tethered, and the internalization signal "presented" in an opposite direction. Though this ultimately might not be a problem, it would be worth trying to create a construct that could function as a fusion to the C-terminus of the cytoplasmic domain.

Second, interactions with the TfR cytoplasmic domain might not naturally be strong enough as a single internalization signal. Instead of artificially generating a string of internalization signal repeats, I would propose that a dual signal motif, specifically the addition of a second YTRF at position 31-34 that has been demonstrated to be effective at doubling the efficiency of TfR endocytosis (Collawn et al., 1993), be used as "bait" in a 2-hybrid assay. Not only should this increase the probability of capturing an interactive partner, but would fairly represent a protein that is known to be functional in living cells.

In conclusion, the findings presented here support a model which states that distinct saturable components of the endocytic machine are required for the recognition of different tyrosine-based internalization signal motifs. It is possible that recognition of the TfR by sorting connectors requires aggregation, encouraged by tight turn structures, to form the necessary high affinity binding site. Alternatively, interaction may only be possible when it is tethered to the 2-dimensional plane of the plasma membrane. Regardless, the identification of other proteins involved in the process of clathrin-mediated endocytosis will be necessary for the proper construction of events involved.

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