Distinct Saturable Pathways for the Endocytosis of Different Tyrosine Motifs

(Received for publication, January 27, 1998, and in revised form, April 2, 1998)

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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 iso-leucine and leucine (dileucine motif). Although 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, and the epidermal growth factor receptor. 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 low-density lipoprotein receptor, epidermal growth factor receptor, 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.

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 Refs. 1–3). At least two types of internalization signals have been described: the tyrosine-based motif and the dileucine based motif. Probably the most thoroughly examined of these is the tyrosine-based motif, which consists of a sequence of 4–6 amino acids, specifically containing a tyrosine that is crucial for proper endocytic targeting (4–9). This tyrosine, which usually resides in a YXXΦ or NPXY motif (where Φ is any amino acid and Θ is a hydrophobic residue), has been shown to capable of forming a tight turn in secondary structure (10–13).

The mechanism behind the sequestration of surface receptors in coated pits remains largely unknown. Some receptors, like the EGFR1 and insulin-R, require ligand binding before they are concentrated into clathrin-coated pits and internalized. Other receptors, including the transferrin receptor, LDLR, and asialoglycoprotein receptor, are constitutively recycled, and spend a majority of their cell surface time clustered in coated pits (reviewed in Refs. 14). In both cases, sorting signals have been suggested to mediate interactions between the receptor and AP2 complexes present in the coated pit (15). These AP2 associations, which have been shown in vitro for a number of different receptors (16–19), would serve either to trap receptors in clathrin-coated pits, or ensure that they are present at the location where new clathrin-coated pit structures are being assembled.

In previous work (20), we have shown that overexpression of a Flag epitope-tagged version of the constitutively endocytosing TIR (tTIR), can lead to saturation of endocytosis for the TIR, but does not affect the rate of internalization of the triggered EGFR. This result supports in part, work by Wiley in 1988 (21), 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 TIR 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 EGFReBS 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, p Alter—, was muta-
genized with the oligonucleotide, PAXbol (5’-agtattcatagtgtcacc-3’), which adds a second XhoI site to the end of the multiple cloning region, and the oligonucleotide AmpRep (5’-gtggctcatgtcgcagcaggg-3’), which repaired the damaged ampicillin-resistance gene, which is in turn

* This work was supported by National Institutes of Health Grant DK40608. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
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used for selection. The resulting plasmid, pAlterXX, was used as an intermediate vector for cloning receptor DNAs into the pUHD10-3 vector (22).

To create the LDLR/pUHD10-3 construct, the HindIII-HindIII fragment from LDLR/pTFZ1 was cloned into the HindIII site of pAlterXX. The resulting LDLR/pAlterXX was then used to isolate the XhoI-XhoI fragment containing the LDLR sequence, and subsequently cloned into the XhoI site of pUHD10-3. The EGFR/pUHD10-3 construct was made by first cloning the XhoI-SalI fragment from EGFR/pBS into pAlter—

and then mutating the EGFR/pAlter—plasmid with oligonucleotides PAXbaI and AmpRep as described above. This EGFR/pAlterXX plasmid was then used to isolate a XhoI-XhoI fragment containing the EGFR sequence, which was then cloned into the XhoI 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 (20). 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 co-transfected 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 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 aLDR (C7) antibody was obtained from ascites from the C7 cell line available through ATCC. The purified monoclonal mouse aEGFR (538) antibody was purchased from Santa Cruz Biotechnology. The monoclonal mouse aLamp1 (H4A3) and mouse aLamp2 (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, under contract N01-HD-7-3263 from the NICHD.

Immunodetection—Non-reducing SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (23). Markers used on gels were purchased from Sigma, and included myosin heavy chain (205 kDa), β-galactosidase (116 kDa), phosphorylase a (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 (Life Technologies, Inc.) were labeled as described previously (20). 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 mM Tris, pH 8.0 (to neutralize the acid), and protein content determined by OD280 measurement. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining to verify purity and concentration. Once purified, 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 Na125I (DuPont NEN), and 0.003% H2O2. After 5 min at room temperature, 25–50 μl of 2% blue dextran was added, and the protein was separated from unreacted Na125I on a 2%-dextran ton column ( Pierce). Purification and labeling of LDL was performed as described by Goldstein and colleagues (24).

Surface Bindings—Cells grown to 90% confluence (~10^6 cells per well) in 6-well plates, were washed in ice 2 times with cold wash medium (Dulbecco’s modified Eagle’s medium buffered with 20 μM HEPES pH 7.4). Incubation mixes were prepared for either 125I-Tf (35 nM 125I-Tf + 12.5 μM unlabeled Tf), 125I-EGF (2.5 nM 125I-EGF + 200 nM unlabeled EGF), 125I-C7 (21 nM 125I-C7 + 500 nM unlabeled C7), 125I-LDL (10 nM 125I-LDL + 100 nM unlabeled LDL), 125I-HA3A (5 nM 125I-HA3A + 260 nM unlabeled HA3A), or 125I-HA4B (2.6 nM 125I-HA4B + 430 nM unlabeled HA4B). Nonspecific binding measurements were made by including unlabeled ligand or antibody in the incubation mixtures as shown. All bindings were carried out on ice, rocking for 90 min, after which they were washed four times in cold final wash (150 mM NaCl, 20 mM HEPES pH 7.4, 1 mM CaCl2, 5 mM KCl, and 1 mM MgCl2), solubilized in 1.5 ml of 0.1 N NaOH, 0.1% Triton X-100, and counted for 1–6 min in a γ-counter.

Uptake Protocol—the rate of uptake was determined as described previously for the ligands 125I-Tf and 125I-EGF (20). Amounts of either 125I-labeled or unlabeled Tf, EGF, and C7 that were used in the uptake assay are as described above. Since our normal acid stripping procedure was only about 30% efficient at removing surface bound C7 antibody, it had to be modified. Instead of a 2-min wash with 3 ml of acid wash (0.2 N acetic acid, 0.5 M NaCl), cells were incubated on ice for 5 min with 3 ml of 30 μg/ml Pronase (Pierce). This stripping procedure removed at least 90% of all surface bound 125I-C7, without affecting the final cell count per well. Internalization rates for all ligands are expressed as number of 125I-labeled molecules internalized per surface receptor per minute.

Immono-Electron Microscopy—Cell lines were fixed in 4% paraformaldehyde and 0.1% gluteraldehyde in 100 mM HEPES pH 7.2 for 30 min at ambient temperature. The fixed cell pellets were rinsed with 100 mM HEPES, infused with polyvinylpyrrolidone and sucrose (25), and prepared for cryosectioning (26, 27). Each section was immunolabeled with sheep anti-TIR IgG (20), followed by mouse anti-goat IgG (Jackson Research Labe), 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 phosphate-buffered saline.

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 over-express the EGFR, LDLR, or a Flag epitope-tagged TIR (tTIR). 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-re-
sensitive expressors of LDLR and EGFR, respectively. Although EGFR8 cells can be maintained in the same growth media as tTfR20-2 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 physical appearance of induced LDLR21 cells supports this theory since numerous fatty vesicles, as determined by Oil Red-O staining (data not shown), are present in the cytosol. In order to circumvent this problem, 10% delipidated fetal bovine serum (prepared as described in Ref. 24) was used in lieu of normal serum in LDLR21 media. Although usage of this media led to the up-regulation of endogenous LDLR and TfR (and possibly others), all subsequent comparisons with the LDLR21 cells were done in identical serum conditions.

Characterization of the tTfR20-2 cells from previous studies showed that expression of tTfR as high as 20-fold over endogenous TfR, leads to significant competition for endocytosis (20). To determine whether induction of the LDLR could saturate its own endocytosis, the rate of internalization for 125I-labeled C7 antibody (mouse IgG2a anti-LDLR) (28) was examined in LDLR21 cells expressing a range of surface LDLR numbers. Beisiegel and co-workers (28) had previously demonstrated that this antibody reacted with a 1:1 stoichiometry with the LDLR and used it to measure the cycling of the LDLR. Use of the C7 antibody instead of the receptor’s natural ligand, LDL, circumvents difficulties of losing LDL activity during radioactive labeling. Varying levels of LDLR expression were accomplished by first growing several sets of cells to maximal expression, then adding tetracycline (2 μg/ml) to individual sets at different times before the experiment was performed (from 0 to 20 h). For each set of cells, a surface binding and uptake assay were performed using 125I-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 (29) showed that cells expressing LDLRs that are unable to undergo clathrin-mediated endocytosis due to a Tyr → Cys substitution in their internalization signal, internalize 125I-Tf at a rate that is 5-fold slower than that seen with normal cells. 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 surface bindings and uptake assays on EGFR8 cells, using 125I-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 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 recycle continuously, 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. tTfR20-2 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 125I-C7. Even though tTfR is expressed at levels shown previously to be sufficient for saturation of TfR internalization (20),
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 125I-Tf internalization (Fig. 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 (20), 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 that were repressed at five different time points, as described in Fig. 2, were used to measure surface bindings (c) with either 21 nM 125I-C7 (cross-hatched bars) or 35 nM 125I-Tf (solid bars). 125I-Tf was also used in an uptake assay (f) on the same set of cells using 21 nM 125I-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: although the rate of Tf internalization in endogenous HeLa cells 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.)

**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 (15, 20). To visualize this and 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 goat anti-mouse IgG conjugated to 10-nm gold. In the absence of fTfR expression (Fig. 4a), endogenous TfR is present on the plasma membrane in small amounts. After 3 days (Fig. 4b) or more than 7 days (Fig. 4b, inset) in the absence of tetracycline, fTfR expression increases dramatically and greater numbers of total TfRs are present on

### TABLE I

**Internalization signals**

| Protein     | Primary Localization | Sorting Signals |
|-------------|----------------------|-----------------|
| TfR Receptor| Early Endosome       | N-terminus      |
| LDL Receptor| Early Endosome       | TM, YFIP6, 72a  |
| EGF Receptor| Plasma Membrane      | Multiple signals|
| Lamp1       | Lysosome             | N-terminus      |
| Lamp2       | Lysosome             | N-terminus      |

**FIG. 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 125I-C7 (cross-hatched bars, see inset for clarity) or 35 nm 125I-Tf (solid bars). 125I-C7 was also used in an uptake assay (b) on the same set of cells. LDLR21 cells that were repressed at five different time points, as described in Fig. 2, were used to measure surface bindings (c) with either 21 nm 125I-C7 (cross-hatched bars) or 35 nm 125I-Tf (solid bars). 125I-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 125I-EGF (white bars) or 10 nm 125I-LDL (cross-hatched bars). An uptake assay (f) was performed on the same set of cells using 21 nm 125I-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: although the rate of Tf internalization in endogenous HeLa cells 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.)
the cell surface. The immunogold label is more homogeneously distributed along the plasma membrane compared with cells lacking fTfR expression. In addition, there is not a significant increase in the amount of coated vesicles proteins in these cells (Fig. 5). These results indicate 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 YXXΦ conformation (30, 31). 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, mutation of the tyrosine from Lamp1 to an alanine residue completely abolishes clathrin-mediated endocytosis of this molecule (32), indicating that the Lamp1 tyrosine motif is indeed an endocytosis signal. Although the tyrosine motif from Lamp2 has not yet been demonstrated as a functional internalization signal, the striking similarity of its cytoplasmic domain to Lamp1 suggests that it is. Finally, overexpression of a chimeric protein that contains a tyrosine-based internalization signal leads to an accumulation of Lamp1 and Lamp2 on the cell surface (15), which implies 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 selected 125I-labeled monoclonal antibodies to either Lamp1 or Lamp2. In fTfR20-2 cells overexpressing the fTfR (Fig. 6a), 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. 6b). Overexpression of LDLR (Fig. 6c), however, does not
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...ume for the FTR 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. A couple different 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 (33), EGF and Tf (34, 35), Tf and asialoglycoprotein (36, 37), Tf and α2-macroglobulin (38), LDL and α2-macroglobulin (39), EGF and α2-macroglobulin (40), and EGF and insulin (41). More recent evidence shows that a heterogeneous population of receptors can be sedimented with a single horseradish peroxidase-conjugated ligand using 3,3′-diaminobenzidine density shift analysis (37, 42). The results showed that horseradish peroxidase/Tf/TfR-containing vesicles copurified with the asialoglycoprotein receptor and mannose 6-phosphate receptor. Together with the double-label studies, these results demonstrate that EGFR, LDLR, TIR, asialoglycoprotein receptor, insulin-R, and mannose 6-phosphate receptor 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 laboratories to try and identify proteins that were interacting with the internalization signal. Results obtained with affinity chromatography (18, 49–51) and coimmunoprecipitation studies (46–49) 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 (19) used the two-hybrid system to isolate the μ2 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. Photoactive peptides containing either this sequence or the Tyr → Ala-mutated sequence SDAQRL have further defined the specificity of the μ2 interaction to the tyrosine residue, and showed a dependence of the interaction on clathrin and phosphoinositides (50). Both the position and context of the internalization signal are determining factors in this interaction (51, 52). 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.

Although 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 laboratories 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 with that of the wild type receptor (49, 53). In addition, previous work by our laboratory and others show that although the TIR and EGFR contain tyrosine-based internalization motifs, they are unable to saturate endocytosis for one another when overexpressed (20, 21). This suggests that TIR and EGFR are saturating different limiting components of the endocytic mechanism.

In the current study, we have demonstrated that the TIR, 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 the endocytic system, no competition is evident between LDLR and TIR, or LDLR and EGFR. The implication, taken together with our previous results which show that the TIR 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
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FIG. 7. Model depicting the role of sorting connectors in clathrin-mediated endocytosis. a, in untransfected cells, sorting connectors for LDLR and TR/Lamp1 are in excess, and available to recruit receptors to the coated pit. b, cells overexpressing the TIR deplete the available TR/Lamp1 sorting connector pool. The competition for TR/Lamp1 connectors leads to an accumulation of both TR and Lamp1 protein on the surface,、“waiting” for free sorting connectors. Endocytosis of the LDLR continues unhindered, since its sorting connector pool is unchanged.

After demonstrating that the TIR, 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 the 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 TIR lead to a significant increase in the number of Lamp1 molecules on the cell surface, indicating that this protein is competing for the same limiting component as TIR. This is in agreement with work by Marks and colleagues (15) who showed that overexpression of a tyrosine-based sorting signal causes a redistribution of TIR, Lamp1, and Lamp2 to the cell surface. We did not find, however, that overexpression of TIR 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? Although interactions with AP2 may describe one pathway, 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. 7). In support of this theory, other proteins besides AP2 have been implicated in the recognition of internalization signals. The Ep15 protein, originally identified as a substrate for the EGFR tyrosine kinase domain, has been shown to associate with α-adaptin from the AP2 complex (54) and the light chain of clathrin (55). 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 β2-adrenergic receptor which contains a tyrosine-based internalization motif (56) and requires the presence of β-arrestin or arrestin-3 for efficient internalization to occur (57). Additionally, β-arrestin has been shown to interact directly with clathrin heavy chain (57–59), suggesting that it may play a role similar to AP2 in β2-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 TIR (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. Alternately, the receptors could be interacting with different domains of the same protein, for example, AP2. This explanation is unlikely to be true for the lack of competition between the EGFR and the TIR or between the EGFR and the LDLR. The saturation of the rate-limiting component for EGFR endocytosis occurs at a much lower concentration of receptors than either the TIR or the LDLR (Fig. 2c).

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J. Biol. Chem. 1998, 273:17056-17063.
doi: 10.1074/jbc.273.27.17056

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