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

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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 125I-transferrin uptake per surface TfR is reduced by a factor of 4 in induced cells. Consistent with endocytosis being the rate-limiting step, TfRs shift from an endosomal to more of a plasma membrane distribution with TfR overexpression. The chlathrin-associated protein AP-2 has been proposed to interact directly with the cytoplasmic domain of many receptors, yet no changes in the amount or distribution of AP-2 were detected in induced cells. The internalization rate for the 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, 125I-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.

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, although 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 (1–9).

Internalization signals have been identified for a large number of proteins that span several different classes of receptors (reviewed in Ref. 9). A common tyrosine-based motif, which has been proposed to form secondary structure comprising a β-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 AP-2 has been proposed to be the primary candidate for interacting with the cytoplasmic domains of receptors targeted for internalization (10–13).

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 (14), we show that by overexpressing an epitope-tagged TfR in HeLa cells, the endocytic mechanism can be saturated such that the rate of internalization for Tf per surface TfR decreases at high TfR concentrations. The expression and distribution of AP-2 remain unaltered in induced cells. Additionally, we demonstrate that although the pathway of endocytosis for Tf is saturated, the activated EGFR continues to be internalized at a rate similar to that in 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) (15). The pUHD10-3 plasmid was a gift from Drs. M. Gossen and H. Bujard (Zentrum für Molekulare Biologie, Universität Heidelberg) (14).

Subcloning and Site-directed Mutagenesis—The human TfR cDNA coding region in pCD-TR1 is contained within a 2.5-kilobase BamHI-XhoI fragment. A HindIII site 900 bases from the start codon was used to generate the 1.5-kilobase HindIII-XhoI fragment, which was subsequently cloned into the M13mp18 vector for mutagenesis. The oligonucleotide-directed mutagenesis system (Amersham Corp.) was used to insert the sequence for the flag epitope (-DYKDDDDK-) (16) 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 rTfR/pUHD10-3 construct.

Iodination—Either 1600 μg of human holo-Tf (Intergen Co.) or 5 μg of human EGF (Life Technologies, Inc.) was used in a 50-μl reaction of 225 mM sodium phosphate buffer (pH 7.0), 0.5 units of lactoperoxidase (Sigma), 2 μCi of carrier-free Na125I (DuPont NEN), and 0.003% H2O2 (Sigma). The mixture was allowed to react for 5 min at room temperature before adding 50 μl of 2% blue dextran in phosphate-buffered saline. The protein was separated from unreacted Na125I on a 2-ml desalting column (Pierce).

Immunodetection—Cells grown on coverslips were washed several times with phosphate-buffered saline, fixed for 15 min at room temperature with 3% paraformaldehyde, and washed an additional two times with phosphate-buffered saline (17, 18). Cells on coverslips were incubated for 1 h at room temperature with a 1:50 dilution of sheep anti-TfR antibody (characteristics similar to goat anti-TF antibody described earlier (19)), followed by a second 1-h incubation at room temperature with a 1:50 dilution of fluorescein isothiocyanate-labeled swine anti-

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1The abbreviations used are: TfR, transferrin receptor; Tf, transferrin; EGF, epidermal growth factor; AP-2, clathrin-associated protein; C-flag TfR, carboxyl-terminal flag epitope-tagged transferrin receptor.

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Identification of Two Clones That Express Epitope-tagged TFR under Tetracycline Regulation—A cell line capable of overexpressing the TFR was needed to study the effect of TIR density on endocytosis. The tetracycline-responsive promoter system developed by Gossen and Bujard (14) 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 Escherichia coli and the VP16 activation domain from herpes simplex virus. In the absence of tetracycline, the tTA is free to bind tetracycline operator sequences found in the promoter region of the pUHD10-3 plasmid to promote transcription. When tetracycline is added to the cell medium, the tTA fusion protein is blocked from interacting with the promoter, and transcription does not occur.

The human TIR sequence containing a flag epitope tag at its carboxyl terminus (C-flag TIR) was subcloned into the pUHD10-3 vector. The TIR/pUHD10-3 construct along with a puromycin selection vector (pBSpac) were cotransfected into tTA-expressing HeLa cells. Approximately 30 colonies were isolated and screened by Western analysis for inducible expression of the C-flag TIR in the absence of tetracycline in the medium. Induction of the C-flag TIR (94 kDa) along with a minor amount of unreduced TIR (180 kDa) was detected in two different clones using the anti-flag antibody (Fig. 1, right panel). Since the promoter region by itself is silent in mammalian cells, no detectable expression of the C-flag TIR is seen at day 0. Anti-TIR antibody shows endogenous levels of wild-type TIR at day 0 of induction and a distinct increase in total TIRs (wild-type and C-flag TIRs) after 3–4 days (Fig. 1, left panel). Clone TFR20-2 was used in subsequent experiments.

Before quantitative 125I-TF uptake measurements of the TFR20-2 clone as a population could be made, we wanted to show that expression of the C-flag TIR was homogeneously induced in all of the cells. TFR20-2 cells were grown in either the absence or presence of tetracycline to represent induced or uninduced cells, respectively. Surface TIR expression was visualized by incubating nonpermeabilized fixed cells with a fluorescein isothiocyanate-conjugated polyclonal antibody to the TIR. A more intense surface fluorescence labeling is apparent in induced cells (Fig. 2, −Tet), indicating a greater number of TIRs. This level of fluorescence was seen uniformly in all cells for every microscopic field observed. Uninduced cells (Fig. 2, +Tet) display a labeling pattern identical to that of the parent TIR-HeLa cell line (data not shown), indicating full repression of the C-flag TIR protein.

Stable TFR20-2 Cells Can Be Induced to Express C-Flag TIR at Levels 10–30-fold over Endogenous TIR—The binding of Tf was examined to quantitate the actual number of TIRs being expressed on the cell surface of the TFR20-2 clone. Scatchard analysis was performed on uninduced and induced TFR20-2 cells (Fig. 3). Uninduced cells show binding sites equivalent to 1.5–2.0 × 10^5 TIRs/cell surface and a Kd of Tf for the TIR of 3.1 nM. Cell-surface binding sites on the order of 2.0–4.0 × 10^6
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Fig. 2. Immunofluorescent detection of cell-surface TfR in induced and uninduced fTfR20-2 cells. fTfR20-2 cells were grown on coverslips in the presence (+Tet; uninduced) or absence (−Tet; induced) of tetracycline in the medium, as indicated, for 96 h. Cells were fixed in 3% paraformaldehyde and sequentially incubated with sheep anti-TfR primary and fluorescein isothiocyanate-labeled swine anti-goat secondary antibodies. The same exposure was taken for both fields to visualize the increase in TfRs in induced (−Tet) cells.

Fig. 3. Scatchard analysis of 125I-Tf binding on fTfR20-2 cells. Cells incubated with a range of 125I-Tf concentrations (0.5–12 nM for uninduced cells and 5–120 nM for induced cells) for 90 min at 4 °C were washed four times with phosphate-buffered saline, solubilized, and counted. Results are plotted as amount bound/free versus bound. The calculated values of the dissociation constant (Kd) for uninduced cells (closed triangles) and for cells induced for 7 days (open squares) were 3.1 and 25.8 nM, respectively. The inset shows the same plot for uninduced cells at a different scale for clarity.

TfRs/cell, a factor of 10–20-fold increase over endogenous TfR, are measured in induced cells. The Kd 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 125I-Tf to ensure that the uptake assays reflected the rate of uptake, and not the rate of Tf binding. Bindings performed with either 210 or 420 nM 125I-Tf yielded similar results, indicating that Tf binding was saturating and not the limiting factor in the uptake experiments (data not shown).

Rate of Tf Uptake per Surface TfR Is Decreased in Induced fTfR20-2 Cells—Rates of 125I-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 125I-Tf (210 nM) for 2, 4, 6, or 8 min at 37 °C and 5% CO2. Uninduced fTfR20-2 cells expressing 2 × 106 TfRs/cell surface display an endocytic rate of 0.39 Tf molecules/surface TfR/min (Fig. 4). In induced cells (2 × 109 TfRs/cell surface), the measured rate was 0.10 Tf molecules/surface TfR/min, a significant 3.9-fold decrease. Control uptakes were performed on the parent TA 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 125I-Tf endocytosis was detected (data not shown).

The number of TfRs expressed on the surface was compared with the rate of 125I-Tf uptake per surface TfR 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 six-well plates and adding tetracycline for varying periods of time gave more precise control over TfR number. Immediately following tetracycline addition, the number of TfRs on the cell surface decreased at a rate reflective of the half-life of the receptor. Uptake of 125I-Tf was measured 12–72 h after repressing TfR synthesis. Fig. 5 shows the compiled results from two experiments. Each point represents one uptake assay from which an endocytic rate and surface TfR number were determined. Although the rate of endocytosis of 125I-Tf increases with levels of TfR expression up to 20-fold over endogenous levels, the relationship is not linear, but rather appears to saturate at surface TfR densities >1 million. These results imply that the endocytosis of the TfR becomes limiting at these higher receptor concentrations.

Proportion of TfRs on the Cell Surface Increases with TfR Overexpression—If the TfR is internalized at a slower rate per surface TfR in induced cells and TfR recycling remains the same, then a change in the distribution of receptors from the internal compartments to the cell surface should be apparent. The TfR distribution changes after a 10-fold induction of surface receptor number (from 2 × 106 to 2 × 108 TfRs/cell surface) from a surface/internal ratio of 1:4 for uninduced fTfR20-2 cells to a ratio of 1:1 for induced fTfR20-2 cells (Fig. 6). The surface/internal ratio for uninduced fTfR20-2 cells is identical to the distribution seen in the parent TA HeLa cell line (data not shown). These results are consistent with the idea that TfRs recycling back to the plasma membrane at a normal rate will have to wait for a longer period of time to be internalized by a saturated endocytic system.

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 EGFR. The EGFR has been shown to colocalize with the TfR in coated pits after binding its ligand, EGF (26, 27), 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 125I-EGF in induced and uninduced fTfR20-2 cells (Fig. 7). Although induced cells express nearly 2 million TfRs on the cell surface, a density that we have shown to impede the endocytosis of the TfR, the rate of endocytosis of the EGFR is not significantly changed from that of uninduced cells (compare 0.17 with 0.16 EGF molecules/
TfRs in the cell.

Surface (internal) CO2. Cells were either solubilized immediately (total) or washed with acid wash prior to solubilization and counting (internal). Internal number of receptors and peptides targeted for internalization came from work that described its involvement. The adaptor protein complex AP-2 has been proposed to be the “recruiter” protein (11). AP-2 is directly involved in the recruitment of clathrin to the membrane and potentiates the formation of clathrin structures. Early evidence that AP-2 interacts with cytoplasmic domains of receptors targeted for internalization came from work that described its

FIG. 5. Saturation of TfR endocytosis relative to receptor number. TfRp2-2 cells were induced to overexpress the TfR and then repressed with tetracycline for 0, 12, 24, 48, and 72 h to generate a range of TfR numbers. Individual uptake assays were performed 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.

FIG. 6. Distribution of TfR in induced and uninduced cells. Cell-surface TfRs were determined by incubating cells with medium containing 210 nm 125I-Tf for 90 min at 4°C, followed by solubilizing and counting. For internal and total receptor determinations, cells were first pre-equilibrated with 210 nm 125I-Tf for 30 min at 37°C and 5% CO2. Cells were either solubilized immediately (total) or washed with acid wash prior to solubilization and counting (internal). Internal (black bars) and surface (grey bars) TfRs are plotted as a percent of the total TfRs in the cell. Error bars were determined from the mean of two experiments.

FIG. 7. Comparison of rates of internalization of EGF for induced and uninduced cells. Uptake assays were performed using 2.5 nm 125I-EGF on uninduced (closed triangles) and induced (open squares) cells for several different time points. The calculated rate of EGF uptake, EGFR number, and TfR number for uninduced cells are 0.17 EGF molecules/surface EGFR/min, 3.00 \times 10^4 EGFRs/cell surface, and 1.80 \times 10^5 TfRs/cell surface, respectively. For induced cells, these values are 0.16 EGF molecules/surface EGFR/min, 3.00 \times 10^4 EGFRs/cell surface, and 1.87 \times 10^5 TfRs/cell surface.

FIG. 8. Lack of AP-2 redistribution to cellular membranes. Uninduced (+Tet) and induced (–Tet) fTfR20-2 cells were freeze-fractured to expose the cytoplasmic face of the lipid bilayer. Fractured cells were treated with either Buffer E (AP-2 intact) or Buffer F (AP-2 removed) prior to scraping wells to remove attached membranes. The pelleted membranes (P), supernatants (S), and whole cell lysates, each representing equal portions of sample, were run on reducing SDS-polyacrylamide (8%) gels; transferred to nitrocellulose; and probed with antibodies to either AP-2 or TfR.

Distribution of the Clathrin Adaptor Protein AP-2 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 (1, 5, 6, 28, 29). AP-2, a major component of the clathrin-coated pit, has been shown to interact specifically with a number of receptors and peptides targeted for internalization (12, 30–36). Using electron microscopy to quantitate their results, both Iacopetta et al. (28) and Miller et al. (37) 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. If more clathrate structures are associated with the plasma membrane, then more AP-2 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 AP-2 to the plasma membrane, as might be expected if AP-2 interacts with the cytoplasmic domain of the TfR. The amount of AP-2 either attached to the membrane or present in the cytosol was measured in both uninduced and induced cells to determine the AP-2 distribution (Fig. 8). Using a protocol modified from Mahaffey et al. (25), cells were freeze-fractured to expose the cytoplasmic face and thawed in either a control buffer (Buffer E), which preserves AP-2 and clathrin binding to membranes, or a Tris buffer (Buffer F) to dissociate clathrin and adaptor proteins from the membrane and to show the total amount of AP-2 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 AP-2 or TfR. The levels of AP-2 seen in the membrane (pellet) fractions of uninduced or induced fTfR20-2 cells washed with Buffer E are similar. No AP-2 was detected in membranes washed with Buffer F, indicating that AP-2 could be removed from the membranes as described previously (25). A direct comparison of whole cell lysates demonstrated that the overall levels of AP-2 expression remain unchanged, regardless of the number of TfRs. We conclude that AP-2 is not actively recruited to the pla
specific association with an affinity matrix column composed of the internalization signal-containing cytoplasmic domains of either the low density lipoprotein receptor or the mannose 6-phosphate receptor (11, 33). The ability of the cytoplasmic domains of internalizing receptors (low density lipoprotein receptor, mannose 6-phosphate receptor, and immunoglobulin receptor) to elute AP-2 from the affinity column lent support to the idea that AP-2 was a common component of the endocytic mechanism. Co-immunoprecipitation studies revealed a strong interaction between AP-2 and activated EGFR (38, 39). In contrast, recent evidence suggested that the high affinity binding of AP-2 to the EGFR was not necessary for efficient internalization of the EGFR (30, 32). No measurable binding of AP-2 to the cytoplasmic domain of the TfR has been demonstrated, although interactions between the tetrapeptide internalization sequence of the TfR and the μ-subunit of AP-2 have been detected using the two-hybrid system and surface plasmon resonance (13).

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 demonstrates that overexpression of the TfR does saturate some rate-limiting step or component of its own endocytosis. In uninduced TfR20-2 cells as well as in the parent TTA HeLa cell line, an average of $1.50 \times 10^7$ TfRs are on the cell surface, and Tf is internalized at a rate of 0.39 Tf molecules/surface TfR/min. When TfR20-2 cells are induced, the surface TfR population increases to between 2 and $4 \times 10^8$ surface TfRs/cell, and a decreased rate of as low as 0.10 Tf molecules internalized per surface TfR/min 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 is 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 TfRs have been published. Iacopetta et al. (28) used electron microscopy to demonstrate that high TfR densities led to an increase in the number of coated pits forming at the plasma membrane. Miller et al. (37) 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. These results imply that receptors recruit AP-2 and clathrin to the plasma membrane. We did not detect any redistribution of AP-2 from the cytosol to the plasma membrane or any increase in AP-2 or clathrin concentrations (data not shown) in cells under conditions in which we see comparable numbers of TfRs (the induction of TfR is 10–20-fold and the rate of $^{125}$I-Tf uptake per surface TfR is decreased 4-fold). Second, Rothenberger et al. (40) reported that no changes in the rate of $^{125}$I-Tf endocytosis per surface TfR were detected in mouse cells expressing large numbers of human TfRs ($4.6 \times 10^6$ versus lower numbers of TfRs ($1.5 \times 10^5$), although the uptake rate of $^{59}$Fe per TfR decreased. They concluded that exocytosis rather than endocytosis slowed at high TfR numbers. No changes in the rate of exocytosis of the TfR were detected in our system. 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 AP-2 with the plasma membrane has been shown by several groups to reflect an accurate AP-2 distribution (12, 25, 41). The method of measuring $^{125}$I-Tf uptake differs from that of Rothenberger et al. (40) 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 et al. 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 AP-2 to the plasma membrane is consistent with recent work by Santini and Keen (42). Using an immobilized antigen for IgE Fc receptors, they were able to show receptor activation without internalization, which led to an aggregation of receptors at the exposed surface. No discernible difference in the level or distribution of either clathrin or AP-2 between activated and inactive receptors was detected. Together with our data, this suggests that AP-2 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 (43). 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 EGF in the coated pits could be increased, and the rate of internalization measured. Although saturation of the endocytosis of the EGFR was obvious, the rate of TfR internalization was unaffected. Studies of Lamaze et al. (44) demonstrated that endocytosis of the EGFR and the TfR has different biochemical requirements.

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. In addition, 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 AP-2 remain 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 (45), the asialoglycoprotein receptor (46), the low density lipoprotein receptor (5, 36), and vesicular stomatitis virus protein G (47). Many membrane proteins have multiple possible endocytic signals, such as the insulin receptor β-subunit, which has two tyrosine-based signals in tandem (48). The cation-dependent mannose 6-phosphate receptor has a tyrosine-based as well as a dileucine-based sorting motif (49, 50), as do the EGFR (51) and the glucose 4 transporter (52). Often, the signals that 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 to quantitatively look for competition in vivo with unaltered endogenous receptors.

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