Quantitative Analysis of the Endocytic System Involved in Hormone-induced Receptor Internalization*

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We have developed a quantitative method to evaluate the interaction between cell surface receptors and the endocytic apparatus. This method exploits occupancy-dependent changes in internalization rates that occur in cells expressing high numbers of receptors. We found that constitutive internalization of the transferrin receptor behaves as a simple, first order process that is unaltered by ligand. Internalization of the epidermal growth factor (EGF) receptor, however, behaves as a saturable, second order process that is induced by receptor occupancy. Internalization of EGF receptors occurs through at least two distinct pathways: a low capacity pathway that has a relatively high affinity for occupied receptors, and a low affinity pathway that has a much higher capacity. The high affinity pathway was observed in all cells having receptors with intrinsic tyrosine kinase activity. Mutant EGF receptors lacking kinase activity could not utilize the high affinity pathway and were internalized only through the low affinity one. Mutated receptors with decreased affinity for kinase substrates were also internalized at decreased rates through the high affinity, inducible pathway. In the case of vitellogenin receptors in Xenopus oocytes, occupied receptors competed more efficiently for internalization than empty ones. Insulin increased the endocytic capacity of oocytes for vitellogenin receptors. Similarly, serum increased the capacity of the inducible pathway for EGF receptors in mammalian cells. These data are consistent with a model of internalization in which occupied receptors bind to specific cellular components that mediate rapid internalization. Ligand-induced internalization results from an increase in the affinity of occupied receptors for the endocytic apparatus. Hormones can also indirectly regulate endocytosis by increasing the number of coated pits or their rate of internalization. The ability to dissect receptor-specific effects from cell-specific ones should be very useful in investigating the molecular mechanisms of receptor-mediated endocytosis.

It has been a quarter of a century since Roth and Porter (1) first described coated pits and postulated that these specialized membrane regions mediate rapid endocytosis of adsorbed molecules. Abundant experimental evidence now supports the hypothesis that receptor-mediated endocytosis proceeds through coated pits. The essential features of the process include ligand binding to specific cell surface receptors and association of ligand-receptor complexes with coated pits, followed by their rapid internalization (2–5). Descriptions of the chemical, physical, and kinetic properties of ligand-receptor interactions are abundant. However, subsequent association of receptors with coated pits is understood only at a descriptive, semiquantitative level.

Differences in receptor-coated pit interaction are thought to lead to differences in the endocytic behavior of various receptors. Receptors whose primary function is bulk transport of nutritional molecules (e.g. transferrin and low density lipoprotein) cluster about coated pits in the unoccupied state. Ligand binding does not lead to their redistribution on the cell surface nor increase the rate of receptor internalization (6–9). In the case of ligand-induced internalization, characteristic of hormone and growth factor receptors (e.g. EGF® and insulin), the ability to cluster in coated pits requires ligand binding (10–13). The functional significance of induced internalization is not entirely clear, but it is thought to be an important regulatory event. Endocytosis may serve to attenuate signal transmission from the cell surface by decreasing receptor density (down-regulation). Alternatively, but not necessarily exclusive of down-regulation, occupied intracellular receptors may generate some responses that cannot arise from the cell surface.

We have been investigating the mechanisms involved in the occupancy-induced internalization of the EGF receptor for several years. In most cells, this process behaves as a rapid first order process (14, 15), indicating that occupancy of the EGF-receptor itself is the rate-limiting step in the pathway. However, this observation reveals little regarding the mechanisms involved. In a series of studies, we have eliminated some hypothetical mechanisms of EGF-induced internalization, such as receptor occupancy directly inducing coated pit internalization, but other models still remain. These mechanisms include receptor-receptor aggregation, release of occupied receptors from a previously "tethered" state and occupancy-induced receptor binding to coated pit proteins. Recently, we have observed that EGF receptor internalization does not appear to be a simple first order process in cells that express high surface receptor densities. Instead, the specific receptor internalization rate progressively diminishes with increasing occupancies, finally reaching a stable minimum value (15, 16). We have also observed that internalization of

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§ The abbreviations used are: EGF, epidermal growth factor; VTG, vitellogenin; TF, transferrin, k, endocytic rate constant; IC, internalization component; λ, coated pit internalization rate constant; K, coated pit constant; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
the vitellogenin receptor in *Xenopus* oocytes, which also expresses high receptor densities, behaves in a similar manner (17). Based on these data, we have suggested that there is a specific "internalization component" in coated pits to which occupied receptors bind and that high levels of occupied surface receptors effectively saturate the endocytic apparatus (15, 17).

In this report, we critically test this model of receptor-mediated endocytosis. We first develop a mechanistic model that describes the interaction between receptors and coated pits that defines the total capacity of the endocytic system. We then experimentally test the predictions implicit in this model using receptors altered by site-directed mutagenesis. Our results demonstrate that induced internalization behaves as a saturable, noncooperative, second-order process exactly as predicted by the model. Using the techniques we developed to analyze our model, we also show that hormones can regulate both the affinity and capacity of the endocytic system for receptors.

### Experimental Procedures

#### Materials

Mouse EGF was purified from submaxillary glands according to the method of Savage and Cohen (18). Human diphtheria Tfn (Calbiochem-Behring) was iron loaded as described (19). Vitellogenin (VTG) was precipitated from estrogen-stimulated female *Xenopus* as described (20). EGF, Tf, and VTG were iodinated using Iodo-Beads (Pierce Chemical Co.) according to the manufacturer's recommendations and free (superscript "") separated from the radiolabeled ligands by dialysis or by passing the mixture over a 0.8-mL column of Sephadex G-10 equilibrated with phosphate buffered saline. The specific activity of (superscript "")-labeled EGF was generally between 600 and 1500 cpm/fmol whereas that of (superscript "")-labeled Tf was between 750 and 3100 cpm/fmol. Sodium ipodate (Oragrain<sup>®</sup>, Squibb Diagnostics) was stored as a 25 mg/ml stock solution in dimethyl sulfoxide.

**Cell Culture**—A431 cells were obtained from Dr. Harry Haigler (University of California, Irvine) and grown in Dulbecco's modified Eagle's medium (Flow Laboratories) containing 10% calf serum, 1 mM methotrexate, 1 mg/ml polyvinylpyrrolidone. The relative activities that otherwise preclude the use of iodinated ligands. Binding was initiated by changing to an identical solution containing 0.01 to 2.5 mg/ml (superscript "")-VTG. Samples of nine oocytes each were removed at 7.5-min intervals for up to 30 min, rapidly washed at 0 °C in O-R2, and divided into three groups of three each. Surface-associated (superscript "")-VTG was removed by the stripping procedure previously described (17), and the relative amount of radioactivity associated with the oocyte surface or interior determined by γ counting. All binding and internalization data were corrected for oocyte surface area.

#### Data Analysis

Values for surface bound and internalized ligand were corrected for nonspecific binding and for spillover from the interior and surface of the cell, respectively. The value of k was determined by plotting the integral of surface-associated ligand against the amount internalized and fitting to Equation 3 under "Results" by the method of least squares. Correlation coefficients of internalization plots were generally ≈0.98. Internalization velocity was determined from 2 to 5 min after initiating binding by linear regression analysis. Templates for data correction and calculation of the integral of surface binding and average surface occupancy were created for Microsoft Excel (Apple Macintosh computers). These templates or the spreadsheet formulas used to create them are available upon request.

### Results

#### Derivation of Internalization Plot Equations—Receptor-mediated endocytosis is thought to involve the following three steps (Fig. 6).

\[
L + R \rightleftharpoons LR \rightleftharpoons LRIC \rightleftharpoons LRIC \rightleftharpoons LRZC
\]

The first step is ligand (L) binding to an empty surface receptor (R). The ligand-receptor complex (LR) then binds to the vitellogenin receptor in oocytes, which also express high receptor densities, behaves in a similar manner (17). Based on these data, we have suggested that there is a specific "internalization component" in coated pits to which occupied receptors bind and that high levels of occupied surface receptors effectively saturate the endocytic apparatus (15, 17). In this report, we critically test this model of receptor-mediated endocytosis. We first develop a mechanistic model that describes the interaction between receptors and coated pits that defines the total capacity of the endocytic system. We then experimentally test the predictions implicit in this model using receptors altered by site-directed mutagenesis. Our results demonstrate that induced internalization behaves as a saturable, noncooperative, second-order process exactly as predicted by the model. Using the techniques we developed to analyze our model, we also show that hormones can regulate both the affinity and capacity of the endocytic system for receptors.

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One cannot readily measure the amount of ligand associated with a specific internalization component, however. Instead, it is more convenient to measure total surface-associated ligand. We have previously defined the specific internalization rate of total (tot) cell surface receptors, k, (14, 22), which is equivalent to the following:

\[
k = \frac{[LRIC]}{[LR]} + [LRIC] - [LR]_0
\]

Substituting and integrating Equation 2 yields the following.

\[
[LR]_0 - k_\text{int} \int [LR]_0^t \text{dt} = \int [LRIC]_0^t \text{dt}
\]

This expression can be evaluated approximately by discrete

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1. L. K. Opresko, unpublished observation.
2. Portions of this paper (including part of "Results," Figs. 7–11, and Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
summation (i.e., the trapezoidal rule):

$$
\int_{\text{dt}} [LR]^{n} = \frac{\Delta t}{2} [LR]^{n} (t_0) + 2 [LR]^{n} (t_1) + \cdots + 2 [LR]^{n} (t_{n-1}) + [LR]^{n} (t_n)
$$

Thus, plotting the integral (summation) of surface-associated ligand against the amount internalized should yield a straight line with a slope of $k$. We call this an internalization plot (23).

At steady state internalization, the rate of formation of internalization complexes should be equal to their rate of loss.

$$
k_[, [LR], [IC]] = k_[, [LR, IC]] + k_[, [LRIC]] + \lambda [LRIC]
$$

The terms are $k_,$ for the second order rate constant of “clustering” and $k_,$, $k_,$ and $\lambda$ for the first order rate constants of ligand coming off the complex, complex unclustering, and coated pit internalization, respectively. Equation 6 assumes that the only significant source of internalization complexes (LRIC) is from free ligand-receptor complexes and ignores recycling of complexes and ligand binding to preclustered receptors. Based on published data, these are reasonable assumptions (6, 10, 11, 24). We can rearrange Equation 6 to yield the following.

$$
[LRIC] = \frac{k_}{k_ + k_ + \lambda} [LR, IC]
$$

For convenience, we can lump the rate constants into a single “coated pit” constant.

$$
k_ = \frac{k_}{k_ + k_ + \lambda}
$$

The total number of binding sites for ligand-receptor complexes on the cell surface should remain constant because of the continual recycling of coated pits and their constituent proteins (25–27). Thus the following should be true:

$$
[IC]^{\infty} = [IC] + [LRIC]
$$

Substituting and rearranging Equation 7 yields:

$$
\frac{[LRIC]}{[LR]} = -k_0[LRIC] + k_0[IC]^{\infty}
$$

Multiplying both sides by $\lambda$ gives us the equivalent of the Eadie-Hofstee equation:

$$
\frac{\lambda[LRIC]}{[LR]} = -\lambda k_0 + \lambda V_{\text{max}}
$$

since the velocity of internalization is $v = \lambda[LRIC]$. When all binding sites on a coated pit are associated with ligand-receptor complexes, we have the maximum rate at which cells can internalize by that mechanism, and thus, $V_{\text{max}} = \lambda [IC]^{\infty}$.

A very useful approximation can be made for those cells that have a large number of surface receptors relative to internalization sites. This can occur either naturally in the case of vitellogenin receptors on Xenopus oocytes (23), or as a consequence of gene amplification, such as in the A431 cell line, which has over $3 \times 10^8$ EGF receptors cell$^{-1}$ (28). One can also achieve high surface densities of receptors by transfecting cells with genes encoding a variety of surface receptors (16). In all these cases, at steady state [LR], $\gg$ [LRIC], and thus $k_ = \frac{\lambda[LRIC]}{[LR]}$. Under these circumstances, we can substitute $k_$ for the term on the left side of Equation 11.

There are several notable features of the above equations. Since $k_,$ is a function of the relative distribution of receptors between internalization complexes and the rest of the cell surface (Equation 3), $k_,$ will change as a function of receptor occupancy only if this distribution is changed. This provides a means of specifically testing whether receptor association with coated pits is ligand independent or dependent. We can also distinguish the contribution of a receptor’s affinity for a coated pit ($K_0$) and the cell’s endocytic capacity ($V_{\text{max}}$) from the overall endocytic rate of a receptor (Equation 11). This should allow us to determine the mechanisms by which hormones affect the internalization of receptors. Because the plot generated by Equation 11 is analogous to a Scatchard plot, we can use many of the same approaches used to analyze ligand-receptor interactions to understand receptor-coated pit interactions. We refer to these as Satin plots since they describe saturation of internalization.

Verification of the Utility of the Internalization Plot Technique—Application of our equations first requires accurate determination of the specific internalization rate of receptors, $k_$. Our previous methods of determining $k_,$ required a steady state of binding at the cell surface (14, 22). Since internalization plots describe surface binding as an integral, they should be insensitive to the kinetics of ligand binding and receptor down-regulation. To critically test this assumption, we incubated normal human fibroblasts with $^{125}$I-EGF for 5 min and then switched to medium containing unlabeled ligand for 5 min. The amount of $^{125}$I-EGF associated with either the surface or inside of cells was then determined by acid stripping. Shown in Fig. 1 is the data plotted in standard kinetic format (A) and as an internalization plot (B). Despite the increasing and subsequent decreasing surface ligand binding, the internalization plot of these data is completely linear, yielding a value of 0.16 min$^{-1}$ for $k_,$. This confirms that steady state binding conditions are not required to accurately measure $k_,$ regardless of whether cells are down-regulating their surface receptors.

Constitutive Internalization Is Not Saturable—Constitutive receptor internalization presumably occurs by ligand-independent association of receptors with both coated and non-coated pits that undergo endocytosis. In both cases, Equation 3 predicts that $k_,$ will be independent of the extent of receptor occupancy. To confirm that receptors internalized by a non-inducible, constitutive pathway yield Satin plots with slopes of zero, we examined the behavior of Tf receptors in mouse B18 L cells. Internalization of transferrin receptors is generally thought to be independent of receptor occupancy in most cell types (8, 9). Ligand-induced internalization and down-regulation of Tf receptors have been described in some cells, however (54). To induce the expression of large numbers of Tf receptors that are required to adequately test our model, mouse B18 L cells were grown in iron-deficient serum (19). The values of $k_,$ were then determined at concentrations of $^{125}$I-Tf between 0.5 and $50 \times 10^{-9}$ M. As expected, a Satin plot of Tf internalization yielded a straight line with a slope approximating zero (Fig. 2A). The value of $k_,$ for Tf receptors in these cells was approximately 0.60 min$^{-1}$ at all ligand concentrations. These results are consistent with ligand-independent endocytosis of the transferrin receptor in these cells and demonstrate that Satin plots of constitutive receptor internalization are linear with slopes of zero.

Induced Internalization Is Saturable—Equation 3 also predicts that $k_,$ will vary as a function of receptor occupancy if clustering in coated pits is perturbed by ligand binding. Since saturation of the endocytic system will be most obvious when the number of occupied surface receptors greatly exceeds the number of coated pits, cells with large numbers of receptors...
should provide the best system in which to test this model. We have previously shown that the specific internalization rate of EGF receptors decreases at high ligand concentrations in A431 cells, which express over \(3 \times 10^8\) EGF receptors/cell (15). Therefore, we determined both the specific internalization rate of EGF receptors and the net internalization velocities in that cell type using several ligand concentrations. The results are presented as a Scatchard plot in Fig. 2B. Plotting internalization velocity versus \(k\) yielded a straight line with a negative slope at low surface occupancies, consistent with our model of occupancy-induced receptor binding to coated pits. As we have previously shown (15), the \(^{125}\)I-EGF was removed and replaced with an identical concentration of unlabeled EGF. At 30-s intervals during the 10-min experiment, cell samples were rapidly shifted to 0°C, rinsed, and acid stripped as described under "Experimental Procedures." A, \(^{125}\)I-EGF associated with the cell surface (■) and internal (○) at each time point. B, internalization plot of the same data as in panel A. The slope of the plot at any point is equal to the specific internalization rate of occupied receptors at that time.

### Hormones Can Regulate the Endocytotic Capacity of Cells—
Our results from A431 cells were consistent with our model of occupancy-induced internalization and suggested that serum treatment can increase the capacity of cells to internalize receptors. To determine whether these results were generally true for occupancy-induced endocytic systems, we examined the uptake of vitellogenin in *Xenopus* oocytes. Since we previously showed that there was a decrease in \(k\) for VTG uptake at high ligand concentration, we expected that Scatchard plots of VTG uptake by oocytes would produce straight lines with negative slopes. Since insulin accelerates the rate of VTG internalization without altering receptor number (17), apparently by increasing the number of coated pits at the cell surface (29), Satin plots of VTG internalization should reveal an increase in \(V_{\text{max}}\) in the presence of insulin. We therefore measured \(k\) at multiple VTG concentrations in control and insulin-treated oocytes. As shown in Fig. 3, Satin plots of these data are parallel straight lines with negative slopes. Insulin increased the maximum velocity of VTG internalization from \(6.67 \times 10^{-3}\) receptor cell\(^{-1}\) min\(^{-1}\), These results support our model of induced internalization and show that the net endocytotic capacity of cells can be regulated by hormonal treatment.

### EGF Receptors Are Internalized Through Two Endocytic Pathways—
We have previously proposed that EGF receptors are internalized by two pathways: a constitutive pathway followed by empty receptors and an induced pathway utilized by occupied receptors (14). It seemed possible that non-saturable internalization seen at high receptor occupancies in A431 cells could reflect constitutive internalization (15, and Fig. 2B). To test this hypothesis, we examined the internalization of EGF in mouse B82 L cells that express either normal or mutated human EGF receptors. Initially, we examined B82 cells expressing 150,000 wild-type human EGF receptors/cell using \(^{125}\)I-EGF concentrations ranging from 0.17 to \(18.3 \times 10^{-8}\) M at 37°C for 0–5 min. Surface and internalized \(^{125}\)I-EGF were measured, and the value of \(k\) at each concentration was calculated from the resulting internalization plots. As shown in Fig. 4A, a Scatchard plot of the data was very similar to that obtained from A431 cells. The apparent affinity of the occupied EGF receptors for the endocytic apparatus was the same as in A431 cells (50,000 receptors/cell), but the \(V_{\text{max}}\) was somewhat higher at 27,000 receptors/cell/min. These data suggest that the affinity of occupied EGF receptors for the coated pit pathway is independent of cell type.

It has been suggested that EGF and insulin receptors that lack intrinsic tyrosine kinase activity do not undergo ligand-induced internalization, and thus are internalized only by a constitutive process (31, 32). To examine this issue, we obtained B82 cells transfected with human EGF receptors lacking intrinsic tyrosine kinase activity due to substitution of methionine for lysine at position 721 (B82-M721 cells; 30). Western blots of whole cell lysates from control and EGF treated cells using anti-phosphotyrosine antibodies confirmed that these receptors lacked ligand-induced kinase activity (data not shown). Scatchard plots of EGF binding to cells expressing kinase negative receptors were indistinguishable from those expressing kinase active receptors with respect to curvilinearity and affinity (data not shown). However, a Scatchard plot of \(^{125}\)I-EGF internalization by B82-M721 cells has a slope of zero, indicating that these kinase negative receptors are internalized by a noninducible pathway (Fig. 4A). There is no down-regulation of kinase negative receptors in response to EGF, also suggesting internalization at the same rate as unoccupied receptors (31, 33).

Although kinase negative receptors did not undergo induced internalization, the maximum rate at which B82-M721 cells internalized EGF was still significant and approximately 30–50% that of the B82 WT cells. Shown in Fig. 4B is the
relationship between surface receptor occupancy and velocity of internalization of the kinase positive and negative receptors. Also shown are data obtained from normal human fibroblasts. The relationship between internalization velocity and receptor occupancy is the same in human fibroblasts as in B82-WT cells at low occupancies. We could not occupy sufficient numbers of receptors in human fibroblasts, however, to observe endocytic saturation as in B82 cells. Nevertheless, the value of \( k_0 \) and internalization velocity were determined at each ligand concentration and converted to a Satin plot in which \( k_0 \) is plotted as a function of internalization velocity. B, induced internalization of the EGF receptor is saturable. Confluent monolayers of A431 cells were incubated overnight in the presence (●) or absence (○) of 5% calf serum. Binding and internalization of \(^{125}\)I-EGF was then measured at 1-min intervals for 5 min using \(^{125}\)I-EGF concentrations from 0.02 to 4.2 \( \times 10^{-8} \) M. The values of \( k_0 \) and internalization velocity were determined at each ligand concentration. The values obtained at the highest surface receptor occupancy (indicated by arrows) were excluded from the linear regression analysis of these plots.

![Figure 2](image-url)  
**FIG. 2.** Satin plots distinguish constitutive from induced internalization. A, internalization of the Tf receptor in B82 cells is not saturable. Binding and internalization of \(^{125}\)I-Tf was measured in B82-WT cells at 1-min intervals for 5 min using \(^{125}\)I-Tf concentrations from 0.5 to 50 \( \times 10^{-8} \) M. The values of \( k_0 \) and internalization velocity were determined as described under "Experimental Procedures" and converted to a Satin plot in which \( k_0 \) is plotted as a function of internalization velocity. B, induced internalization of the EGF receptor is saturable. Confluent monolayers of A431 cells were incubated overnight in the presence (●) or absence (○) of 5% calf serum. Binding and internalization of \(^{125}\)I-EGF was then measured at 1-min intervals for 5 min using \(^{125}\)I-EGF concentrations from 0.02 to 4.2 \( \times 10^{-8} \) M. The values of \( k_0 \) and internalization velocity were determined at each ligand concentration. The values obtained at the highest surface receptor occupancy (indicated by arrows) were excluded from the linear regression analysis of these plots.

![Figure 3](image-url)  
**FIG. 3.** Insulin increases the capacity of Xenopus oocytes to internalize VTG. Binding and internalization of \(^{125}\)I-VTG was measured for up to 60 min using \(^{125}\)I-VTG concentrations from 0.01 to 2.5 mg/ml in oocytes incubated overnight in the absence (C) or presence (●) of 5 \( \mu \)g/ml insulin. The values of \( k_0 \) and internalization velocity were determined and plotted as a Satin plot. The values of \( V_{max} \) determined by linear regression were 6.67 \( \times 10^3 \) and 9.84 \( \times 10^3 \) molecules/cell/min for control and insulin-treated oocytes, respectively.

![Graph](image-url)  
Graph showing the relationship between surface receptor occupancy and velocity of internalization of the kinase positive and negative receptors.
Regulation of Receptor Binding to Coated Pits

**Fig. 4.** Kinase negative EGF receptors are internalized only through a constitutive pathway. A, the values of $k_e$ and internalization velocity were determined in B82 cells transfected with normal (WT, ○) or kinase negative (M7*, □) human EGF receptors using $[^{125}]$-EGF concentrations from 0.17 to $18.3 \times 10^{-9}$ M. The value obtained at the highest surface receptor occupancy of the WT cells was excluded from linear regression analysis of this plot. B, kinase negative EGF receptors are internalized at a rapid rate only at high receptor occupancy. Binding and internalization of $[^{125}]$-EGF was measured in human fibroblasts (□), B82-WT (○), and B82-M7* (□) cells for up to 5 min using $[^{125}]$-EGF concentrations from 0.17 to $18.3 \times 10^{-9}$ M. Surface receptor occupancy was calculated as the average number of receptor occupied during the entire period of internalization.

**Fig. 5.** Kinase active EGF receptors are internalized through two endocytic pathways. The values of $k_e$ and internalization velocity were determined in B82-WT (○ --- ○) and B82-F3 (○ --- ○) cells using $[^{125}]$-EGF concentrations from 0.17 to $18.3 \times 10^{-9}$ M. The relative contribution of the saturable and nonsaturable endocytic pathways to the overall endocytic rate was determined by fitting the data to a two component model using the Simplex Algorithm as described in the text. The mean value (±S.D.) of $k_e$ measured for kinase negative EGF receptors is indicated by the bars for comparison.

These data suggest that the requirement of receptor kinase activity for induced internalization is for phosphorylation of a cellular substrate and that autophosphorylation of the carboxyl terminus tyrosines is not essential to the process.

Receptor-mediated endocytosis is thought to require receptor binding to specific components of coated pits. The interaction between occupied receptors and coated pits is a critical intermediate step that couples ligand binding to rapid internalization since receptors that do not cluster in coated pits are internalized at much slower rates (37, 38). Virtually all studies on the determinants of receptor-mediated endocytosis have utilized class II receptors, which are internalized at a rate that is independent of receptor occupancy. Thus, many investigations have focused on the molecular determinants which allow these receptors to bind to coated pits (4). Understanding the mechanisms of ligand-induced endocytosis presents a different set of problems. In the unoccupied state, these receptors are internalized relatively slowly. When occupied, their internalization rates can increase up to 50-fold. Morphological studies have suggested that a change in receptor distribution from a diffuse to a clustered pattern is strongly correlated with ligand-induced internalization, but many mechanisms can be proposed to account for such a change in distribution. Our previous studies have indicated that affinity of occupied receptors for coated pits is an important parameter of endocytic behavior (15, 17). Thus, we sought a way to determine whether induced internalization was mediated by an increase in affinity of occupied receptors for coated pit proteins. We derived a set of equations that describe this model of receptor-mediated endocytosis and which depend on occupancy-dependent internalization rates to quantitatively describe receptor binding to internalization sites. We have defined the affinity and capacity of the endocytic apparatus for receptors in terms of the specific internalization rate, $k_e$, a value we can easily measure. This approach thus provides a practical, analytical method to test our specific model of the molecular mechanisms of receptor-mediated endocytosis.
The first requirement for testing this model was to have an accurate method for determining the specific internalization rate of occupied receptors. The method we have developed, plotting the integral of surface binding versus internalized ligand, is simple to perform, insensitive to fluctuations in surface binding, and can be applied to most ligands. The data obtained from these internalization plots can then be used to construct a Satin plot, which will be straight only if the relationship that we postulate between receptor occupancy and internalization holds true. As we have shown with A431 cells, B82 cells, and Xenopus oocytes, and with EGF as well as VTG receptors, the predicted relationship between internalization velocity and $k_1$ does indeed appear to hold. It should be stressed that the equations we have developed are highly constrained in that the majority of possible mechanisms for induced internalization will not produce such a relationship (see Miniprint for a full discussion). Mechanisms that are incompatible with our data include both receptor-receptor clustering as well as occupancy-induced release of receptors from a previously immobile state. These processes may still occur on the cell surface, but they cannot be rate limiting for endocytosis. The only other mechanism that cannot be excluded on the basis of Satin plots alone is the presence of a small population of “internalization competent” receptors on the cell surface which are distinct from the majority of non-internalizing receptors. We have previously shown by ligand dilution experiments, however, that stable populations of internalization competent receptors do not exist in A431 cells (15). Instead, all surface receptors seem to be equally available for internalization on the time scale of our experiments (1–5 min).

The only hypothesis that is fully consistent with all the data obtained from a variety of systems is a simple one: ligand-induced internalization is due to an increase of receptor affinity for specific binding sites in coated pits. This model is illustrated in Fig. 6. Empty receptors, or those undergoing constitutive endocytosis, are internalized at rates proportional to their localization in either coated or non-coated pits. Ligand binding does not affect localization of receptors that are constitutively internalized, and thus the value of $k_r$ for transforming growth factor (TGF) or kinase negative EGF receptors is independent of ligand concentration. Morphologic data confirms that the distribution of these receptors on the cell surface does not change with occupancy (9, 12, 31). Constitutive internalization can still be rapid and efficient, however. Endocytosis of TF receptors is more rapid than induced internalization of EGF receptors, probably reflecting extensive constitutive clustering in coated pits (39). Our data for kinase negative EGF receptors demonstrate that constitutive endocytosis can also reach a significant net rate if receptor number is very large and ligand concentration is high.

In the case of ligand-induced endocytosis, occupancy increases the affinity of receptors for coated pits, prolonging their residence time within these structures. Since this interaction is second order, endocytosis of these receptors should behave as a second order process, which is precisely what we observed. Human EGF receptors transfected into mouse cells bind to coated pits with identical affinity as in A431 cells, and there was little variation in receptor affinity for coated pits between multiple experiments despite large differences in overall endocytic rates. Thus, localization to coated pits seems to be an intrinsic, physical property of the occupied EGF receptor. The recent identification of a cytoplasmic region outside the kinase domain that is required for receptor internalization and down-regulation is consistent with this model (16). Eliminating the major autophosphorylation sites from EGF receptors, however, results in a decreased affinity for coated pits. Since these receptors also have a decreased affinity for kinase substrates (36), altering the physical structure of a specific internalization component by tyrosine phosphorylation may be required for receptor association. A critical test of this hypothesis requires further characterization of the domains of the EGF receptor involved in endocytosis as well as identification of the internalization component of coated pits.

Although its precise molecular function during induced-internalization is unclear, we verified that the intrinsic kinase activity of the EGF receptor is necessary for effective association with coated pits. This is entirely consistent with the observation that antibodies against phosphotyrosine also prevent induced endocytosis of EGF receptors (31). Intrinsic kinase activity is also required for ligand-induced internalization of insulin receptors (32). Studies that indicated that kinase activity is not necessary for induced internalization were done using high ligand concentrations in cells containing >200,000 receptors/cell (33, 40). The relatively small differences in ligand accumulation they observed in cells expressing either kinase positive or negative receptors are entirely consistent with our data since high levels of receptor occupancy precludes distinction of induced from constitutive internalization.

In contrast to the invariant intrinsic affinity of normal EGF receptors for coated pits, the maximum rate of endocytosis through the induced pathway varied between 8,000 and 30,000 molecules/cell/min, probably due to differences in the cells. Treatment of A431 cells with serum increased their endocytic capacity for EGF, and insulin induced a similar increase in the capacity of oocytes to internalize VTG. Both of these results could be due to an increase in the number of internalization components at the cell surface or an increase in their rate of internalization. Hormonal treatment has been shown to increase oocyte surface area (29) and fluid-phase uptake (17), consistent with an increase in the number of coated pits on these cells. Heterologous regulation of receptor internalization by hormones thus can result indirectly from an increase in cellular endocytic capacity rather than from a
direct alteration in the receptors themselves.

The total capacity of a cell for endocytosis through coated pits is a function of both their rate of internalization as well as the number of internalization components they contain \( (V_{\text{total}} = \lambda [IC]^{n}) \). Approximately \( \frac{1}{3} \) of transferrin receptors are localized to coated pits (9), and thus we can estimate that the value of \( \lambda \) is 1.8 min\(^{-1}\) in B82 cells (25; Equation 3) and that there must be up to \( 1.7 \times 10^{4} \) EGF receptor internalization sites/cell. Since coated pits cover 1 to 2% of surface area, and the surface areas of L cells and coated pits are \( 2.1 \times 10^{-8} \) and \( 3 \times 10^{-9} \) m\(^2\), respectively (41), this corresponds to approximately 12–24 receptor-binding sites/coated pit. This estimate is consistent with the maximum number (10–13) of ferritin-EGF conjugates found in single pits in A431 cells (11, 42).

Occupied EGF receptors that are not specifically associated with coated pits are still internalized through a secondary, nonsaturable endocytic pathway. Since unoccupied EGF receptors are randomly distributed throughout the plasma membrane (11–13), low affinity internalization probably occurs by nonspecific association of receptors with membrane structures undergoing continual endocytosis. Although they cover only 2% of cell surface area, internalization of membrane through coated pits is substantial because they are internalized at a rapid rate. A simple calculation shows that random association of only 2.2% of EGF receptors with coated pits would be sufficient to account for nonsaturable endocytosis. This estimate represents an upper limit, however, since it ignores the contribution of non-coated pits. Endocytosis of surface membrane through smooth pits can also occur at a significant rate (41, 43). Electron microscopy studies have shown that the major role of coatomer clusters in A431 cells is to invaginate smooth plasma membrane, not at clathrin-coated pits (11, 12, 44). Thus, if one assumes random association of receptors with endocytic structures, the rate of constitutive internalization of both coated and non-coated pits is sufficient to explain nonsaturable, low affinity, endocytosis of EGF receptors.

The molecular basis for receptor interaction with unique components of the endocytic apparatus is unknown. Preliminary information indicates that specificity for binding to coated pits resides at least in part in the cytoplasmic domain of receptors. Mutations in these regions disrupt normal receptor interactions with coated pits and alter endocytic behavior (45–48). Tyrosine residues at critical loci are essential for normal endocytosis of some surface molecules (49, 50). No other unifying features have been identified, however (4). Neither have the putative internalization components associated with coated pits been identified. This function has been proposed for clathrin-associated proteins, but no direct evidence from intact cells has been presented (51–53). Satin plots should allow us to evaluate endocytosis of other genetically engineered receptors and thus develop a molecular model of the structural and enzymatic requirements for induced internalization. Using this technique, we can test the hypothesis that endocytosis of different receptors is mediated by their common interaction with a single coated pit component. This is a crucial first step toward understanding the molecular basis of receptor-coated pit interactions. The ability to distinguish receptor-specific and cell-specific properties of endocytic pathways provides an additional advantage over morphologic techniques. A hormone-induced change in endocytosis could result from a change in receptor structure, e.g., by phosphorylation, or by changing either the number of coated pits or their rate of internalization. Satin plots can distinguish between these different mechanisms. There are only a few limitations to this experimental approach. To obtain useful information from Satin plots, enough receptors must be occupied to achieve at least half-maximal saturation of the endocytic pathway (1/\( K_{v} \)). This may be difficult in cells with few receptors relative to internalization components or when ligand binding induces only a small change in receptor affinity for the endocytic system. Ligand on the cell surface must also be efficiently separated from internalized ligand, and the slope of internalization plots will equal \( k \) only during intervals in which internalized label is not lost from inside the cell. Despite these restrictions, Satin plots remain a useful analytical tool under many circumstances. In particular, it is a very suitable approach for transfected cells in which the number and structure of the receptors is under experimental control. Transfected cells with altered receptors should also enable us to define regions of transmembrane receptors that mediate regulatory and signal transducing functions. This will be an important step toward understanding the biologic significance of ligand-induced internalization.

Acknowledgments—We thank John McParlane for excellent technical assistance during this project. We also thank Drs. Douglas Lauffenburger, Byron Goldstein, and Carla Wofsy for their many useful discussions and comments.

REFERENCES

Regulation of Receptor Binding to Coated Pits


SUPPLEMENTAL MATERIAL TO:
QUANTITATIVE ANALYSIS OF THE ENDOCYTIC SYSTEM INVOLVED IN HOMING-INDUCTED RECEPTOR INTERNALIZATION

By: Kirk A. Land, Lee K. Opresko, Cindy Starkbusch, Brenda J. Walsh, and H. Steven Wiley

Critica for Selection of Models for Receptor-Mediated Endocytosis

Introduction: Kinetic analysis combined with mathematical modeling is a powerful technique for the investigation of receptor endocytosis. Mathematical models are excellent tools for representing hypothetical in a mechanistic and testable form. The evaluation of these models require first defining the physical events which occur and then defining their behavioral characteristics. Often, a model's characteristic feature is inconsistent with experimental data and the model can be discarded a priori. If model behavior and data are consistent, the next step is development of techniques to measure model rate parameters and constants. The final step is to critically test the model by comparing computer-generated simulations against experimental data. In this supplement, we provide examples on the parameters of our current models for late endosome internalization. We also include a description of models which were eliminated on physical as well as model which were only partially described by the experimental data.

Clase of Models and Predicted Behavior: A number of models have been proposed to describe the mechanism of receptor-mediated endocytosis. Most have been developed to test a small set of qualitative observations. In contrast, we have been interested in developing models which can be quantitatively tested against all set of experimental data. Thus our models consist of a series of late signaling steps which can be sequentially observed. To choose an appropriate model, we first define the rate-limiting steps involved, determine the conditions under which these steps can be experimentally observed, and then decide whether the prior limiting steps are consistent with experimental observations. A final model is comprised of the sequence of steps which are consistent with experimental observations. If a proposed step is inconsistent with observations, then the model is discarded. If a step is not rate-limiting for internalization, or cannot be made rate-limiting under experimental conditions, then it is automatically excluded. This does not mean that the process does not occur, but it may be a regulatory step.

Many different types of experimental observations can be used to test a model, but those that most severely constrain model behavior provide the most rigorous tests. Presented below is a list of different rate-limiting steps that have been proposed to be involved in receptor-mediated endocytosis. We classify these steps with respect to their predictions on the relationship between receptor occupancy and internalization since this highly constrains model behavior. These predictions are then compared with the experimental observations.

Rate-limiting steps in Receptor internalization:-Proposed rate-limiting steps in receptor-mediated endocytosis include the following: 1) occupation of surface receptors, 2) ligand-induced receptor un-dimerization, 3) ligand-induced receptor un-binding from an intracellular site on the cell surface, 4) ligand-induced signaling or trigger receptor pools, 5) receptor-induced conformational changes, 6) induction of coiled-coil formation by occupied receptors, 7) diffusion of occupied receptor into coated pits, 8) invasion of a single class of binding sites in coated pits, 9) degradation of raw materials for coated pit formation, 10) binding to and internalization with their components in coated pits. Most models include several of these steps, but we will first examine them individually.

Class I: Increasing receptor occupancy has no effect on receptor-mediated endocytosis. All models in which internalization is a first-order process predict that increasing receptor occupancy has no effect on receptor-mediated endocytosis. This is shown at Figs. 7A. By definition, all receptors participating in a first order internalization process do not interact functionally. Thus for this class of models, receptor occupancy cannot affect the initialization of other receptors either directly or indirectly. Specific types of Class I models include those in which ligand binding occurs in an irreversible step, release of receptors from cytoskeletal attachments or uncovering of 'cryptic pools' of receptors.
Regulation of Receptor Binding to Coated Pits

Class III: Increasing receptor occupancy enhances receptor-mediated endocytosis. All models in which internalization is a cooperative process predict that increasing receptor occupancy enhances receptor-mediated endocytosis. This behavior is illustrated in Fig. 2. In this class of models, the specific internalization rate (k_{int}) increases as the formation of internalization-complexes proceeds. The shape of the cooperativity curve depends on the specifics of the model. Specific class III models include those in which dimensionality is a rate-limiting step for internalization as well as those in which occupied receptors serve as nucleation sites for the formation of coated pits. Thus, increasing receptor occupancy induces receptor-mediated endocytosis. The general behavior of Class III models is shown in Fig. 7C. Models that fall into this category are diverse and deserve a more detailed evaluation.

A. Non-specific induction of endocytosis caused by receptor concentration. Scott, Matthews, and coworkers (47) propose that slowed mobility of the EGF receptor within the lateral plane of the membrane due to formation of receptor aggregates at high receptor densities causes a decrease in specific internalization. This hypothesis assumes that internalization into coated pits is the rate-limiting step for internalization and thus receptor aggregation interferes with endocytosis at the level of movement into coated pits.

B. No rate-limiting step of coated vesicles, one which is internalization dependent. The existence of a minor class of high affinity receptors which is internalized rapidly and a major class of low affinity receptors which is internalized slowly is an example of a process which gives the appearance of negative cooperativity. Reports of high and low affinity receptor subpopulations are prevalent in many receptor systems, including EGF (54), epidermal growth factor (EFG) (55), and insulin (56). The rate-limiting step in this scenario is binding to high-affinity receptors. Class III behavior will result because only the specific internalization rate of high-affinity receptors will be observed at low ligand concentrations. Specific internalization rates will decrease as occupancy of low affinity binding sites increases.

C. Inhibition of endocytosis resulting from the depletion of adaptor pools required for the formation of coated pits. Perry et al. propose that receptors compete for specific components of coated pits (termed "adaptors"). Inhibition of adaptors results in dwell time increased in coated pits by depletion of adaptor pools required for the formation of coated pits. This is one of several models proposed by Ping (53).

D. Saturation endocytic system resulting from the binding of coated receptors to specific (non-diffusional endocytosis) components of coated pits. This is the primary rate-limiting step in the model developed and described in the text. The identity of the rate-limiting components could be adaptors (as in Class III above), but inhibition of internalization is proposed to occur by saturation of low affinity sites with coated pits. Note that only this model predicts that the specific internalization rate will approach a non-zero (constant) value at high occupancies due to the inclusion of an alternate (first-order) pathway for the internalization of occupied receptors that are excluded from the induced pathway.

Analysis of Class III models. Since models of class III predict the saturation of internalization observed in cells expressing the VEGF-R, we explored these models in further detail. The models lack the interpretation of whether differences within the lateral plane of the membrane in rate limiting for endocytosis have been addressed previously (67). This analysis indicates that the lateral diffusion coefficient of the EGF-R is lower than expected based on experiments that do not necessarily seem incompatible with our observations. Therefore, the basal diffusion coefficient of EGF-R is not consistent with current experimental data. In an earlier publication (10), we ruled out the presence of multiple classes or receptors that differed in their internalization process (class IIIb) by showing that internalization of previously occupied receptors could be blocked by the addition of unoccupied ligand. Thus, the model necessary for a receptor equilibrium with the endocytic apparatus is one that may require for ligand binding allosteric transitions in class IIIb. Current data is generally consistent with class III models, so we explored these models in more detail.

Experimental and Development of a Class III Model. Prior to evaluating any model that also our conclusions, it is more revealing to examine a model which has been previously proposed based on experimental data. Since the class III model suggests that increasing receptor occupancy can depolarize the components responsible for assembling coated pits, we next consider an example that suggests that coated pits act as nucleation sites for the formation of coated pits. Evidence that transferrin receptors may provide nucleation sites for coated pits has been presented recently (68). To be consistent with our experimental data, these membranes must be a point at which proteins are assembled in an ordered structure by high receptor occupancy. These two observations form the basis of the models evaluated below. We assume that the components which are depleted are adaptor proteins, but the presence of any other rate-limiting assembly components would produce the same effect.

Controlling mechanisms and key assumptions. Figure 8 illustrates the subcellular and intercellular events which are included in our "Adaptor Protein" (AP) model. Saturation of internalization could occur in this scenario for two of our assumptions: 1) depletion of adaptor pools required for the formation of coated pits or 2) saturation of coated pit binding sites (adaptors) at high receptor occupancies. We will examine the influence of these mechanisms by varying the ratio of the total number of adaptor pools (AP) to the total number of receptors (R), the value referred to as Table 1 contains the key model equations; Table 2 contains model parameters used in simulating computer simulations.

<p>| TABLE 1: Equations for the Adaptor Protein Model |</p>
<table>
<thead>
<tr>
<th>Changing pool</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell surface receptors</td>
<td>dR/dt = k_{on}R - k_{off}R</td>
</tr>
<tr>
<td>Internalized receptors</td>
<td>dR_{int}/dt = k_{on}R - k_{off}R - k_{int}R</td>
</tr>
<tr>
<td>Cell surface complexes</td>
<td>dC/dt = k_{on}R + k_{off}R - k_{int}R</td>
</tr>
<tr>
<td>Cell surface dimers</td>
<td>dD/dt = k_{D}R_{int} - k_{D}R</td>
</tr>
<tr>
<td>Dimer with two bound adaptors</td>
<td>dA/dt = k_{A}R_{int} - k_{A}R</td>
</tr>
<tr>
<td>Monomers in coated pit</td>
<td>dM/dt = k_{M}R_{int} - k_{M}R</td>
</tr>
<tr>
<td>Adaptor protein</td>
<td>dAP/dt = k_{AP}R_{int} - k_{AP}R</td>
</tr>
<tr>
<td>Number of coated pits</td>
<td>dN/dt = k_{N}R_{int} - k_{N}R</td>
</tr>
<tr>
<td>Internalized ligand-receptor complexes</td>
<td>dLIC/dt = k_{LIC}R_{int}</td>
</tr>
<tr>
<td>Internalized dimers</td>
<td>dD_{int}/dt = k_{D_{int}}</td>
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</tbody>
</table>

<p>| TABLE 2: Estimated AP model parameter values and rate constants |</p>
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Estimate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>k_{on}</td>
<td>Association rate constant, ligand binding</td>
<td>1.2 x 10^{-5} M^{-1} s^{-1}</td>
<td>99</td>
</tr>
<tr>
<td>k_{off}</td>
<td>Dissociation rate constant, ligand binding</td>
<td>1 x 10^{-8} s^{-1}</td>
<td>99</td>
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<tr>
<td>k_{int}</td>
<td>Equilibrium dissociation constant (k_{on}/k_{off})</td>
<td>3.0 x 10^{-5} M</td>
<td>99</td>
</tr>
<tr>
<td>k_{D}</td>
<td>Clustering rate constant</td>
<td>3.8 x 10^{-1} s^{-1}</td>
<td>99</td>
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<tr>
<td>k_{A}</td>
<td>Unclustering rate constant</td>
<td>1.2 x 10^{-1} s^{-1}</td>
<td>99</td>
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<tr>
<td>k_{K}</td>
<td>Internalization rate constant</td>
<td>1.0 x 10^{-4} s^{-1}</td>
<td>99</td>
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<tr>
<td>k_{M}</td>
<td>Internalization rate constant</td>
<td>1.0 x 10^{-4} s^{-1}</td>
<td>99</td>
</tr>
<tr>
<td>k_{LIC}</td>
<td>Equilibrium dissociation constant, ligand binding</td>
<td>3.0 x 10^{-5} M</td>
<td>99</td>
</tr>
<tr>
<td>k_{N}</td>
<td>Equilibrium dissociation constant, ligand binding</td>
<td>1.0 x 10^{-4} s^{-1}</td>
<td>99</td>
</tr>
<tr>
<td>k_{AP}</td>
<td>Equilibrium dissociation constant, ligand binding</td>
<td>1.0 x 10^{-4} s^{-1}</td>
<td>99</td>
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<tr>
<td>k_{D_{int}}</td>
<td>Equilibrium dissociation constant, ligand binding</td>
<td>1.0 x 10^{-4} s^{-1}</td>
<td>99</td>
</tr>
</tbody>
</table>

*Estimated by examining kinetics of formation of a single pit. (Let = number of polymerization sites. When /= 1, l = 1, AP, where l is the number of available sites. Assuming coated pit formation as a circle underlies the cell membrane which stabilizes once a critical number (N) of APs have polymerized, the number of available sites, l, can be approximated as the diameter of the coated pit/unit length per site or &. Substituting this expression for & into the equation for the change in the number of polymerized sites with time, we can solve for k_{LIC} = k_{LIC} AP, assuming that L = constant. & = k_{LIC} AP for the formation of a single pit and & = k_{LIC} AP for assigning the formation of a single pit and & = k_{LIC} AP for assigning the formation of a single pit.

Theoretical predictions and discussion. Figure 9 contains computer-generated k_{off} vs. average surface receptor occupancy curves for our AP model for a range of y values. The values heights of these simulations are as follows: The specific internalization rate increases with receptor occupancy at low occupancies. This is the same behavior typical of a Class III model and results from the saturation of the coated pit nucleation sites in our AP model. Specific internalization rates increase until the supply of adaptor proteins is depleted. The greater the value of y, the greater the increase in the specific internalization rate, thus representing the formation (and internalization) of more coated pits. At low values of y, the internalization rates representing the formation of a very limited number of coated pits on the cell surface. The upper limit on coated-pit number is set by the ratio of AP to y. At higher receptor occupancies, specific internalization rates decrease since they represent saturation of coated-pit binding sites formed at lower receptor occupancy due to high numbers of occupied receptors. Once the maximum number of coated pits form (indicated by the peak in k_{off} vs. occupancy plot for a given y), the model exhibits Class III behavior. Removal of the occupancy-dependent nucleation feature of the model occurs in the class III model proposed in the main text. This suggests that EGF receptors are not involved in coated-pit nucleation, an observation supported by the recent data of Benveniste et al. (55). Note that in our model, we assume the presence of coated pit structures with limited internalization rates.

Our experimental data show that ligand-induced internalization of both the EGF-R and the VEGF-R has been observed, and so if these types of mechanisms are involved in receptor mediated endocytosis, they are not rate limiting (and thus
Regulation of Receptor Binding to Coated Pits

FIG. 9. Simulation of the situation of internalization generated from our Adaptor Protein 1 (AP1) model. The theoretical plots of $k_p$ vs. average surface receptor occupancy were derived from our AP model using the random-walk technique. Values for the specific internalization rate constant $k_p$ were determined from the slope of computer-generated simulation runs (internalized ligand vs. surface bound ligand) for a range of ligand concentrations (0-100 000 ng/ml). $k_p$ values are plotted as a function of average surface receptor occupancy during each 5-minute uptake period. All model parameter values and rates constants are contained in Table 2. The values of the rate, $p$ (APF/RAF), was varied between 0.1 and 1 to examine the effect of stoichiometrically-limiting values of adaptor proteins on endocytic saturation.

Cells expressing WT EGF receptor show varying degrees of saturation. Figure 10 shows the relationship between the specific internalization rate and the endocytosis velocity for three different cell types expressing the WT EGF-R. In our experiments, receptor occupancies are constrained by surface expression of receptors, we took data from cells expressing a range of receptor number. These values are all subsets of the curves generated by our total plot equations. Note that different cell types expressing the WT EGF-R show apparent differences in the degree of kinetic saturation, suggesting that saturation depends upon an aspect of which we are not clear. Some simple estimation of Fig. 11 indicates that changes in either the number of internalization components or the rate of control pit internalization could account for these differences. Examination of parameters suggests that these rates change in the same way. These results indicate the need for further experiments.

Electron microscopy reveals that a high level of receptor occupancy almost always show saturation at a higher final value (not shown). Thus, in characteristic of increased in coated plasmatic areas, but not levels of internalization components (compare Figs 10A and 10C). We cannot rule out a rate increase in the number of coated pits, but observations by other investigators suggest that the fraction of the cell surface covered by coated pits is relatively constant. However, the fact that endocytic rate of cells can be strongly influenced by the lack of some component, consistent with the hypothesis that the rate of coated pit internalization is a cell-specific parameter.

FIG. 10. Simulation of dual pathway model. The model presented in Eq. 13 was simulated using a range of parameter values. Different internalization velocities were achieved by varying $k_{10}$ from 0 to 5 per 1000 cells. Each simulation point is for a different value of $k_{10}$. Initial parameter estimates were: $k_{10}$, 0.02 min$^{-1}$; $k_{10}$, 5000 km$^{-1}$/cell; $k_{15}$, 0.05 km$^{-1}$/cell; p, 0.2. A: The value of $k_{10}$ was varied from 2000-20000 km$^{-1}$/cell. B: The value of $k_{10}$ was varied from 5000-20000 km$^{-1}$/cell. C: The value of $k_{10}$ was varied from 0.001-0.01 km$^{-1}$/cell.

Electron microscopy reveals that a high level of receptor occupancy almost always show saturation at a higher final value (not shown). Thus, in characteristic of increased in coated plasmatic areas, but not levels of internalization components (compare Figs 10A and 10C). We cannot rule out a rate increase in the number of coated pits, but observations by other investigators suggest that the fraction of the cell surface covered by coated pits is relatively constant. However, the fact that endocytic rate of cells can be strongly influenced by the lack of some component, consistent with the hypothesis that the rate of coated pit internalization is a cell-specific parameter.

FIG. 11. Cells expressing the WT EGF-R show varying degrees of endocytic saturation. Values of $k_{10}$ were determined as described in Experimental Procedures for a range of 10-100 000 ng/ml. Error bars show the standard deviation determined among the 10 experiments for each cell type. WT cells expressing the WT EGF-R (two different strains) were used and A431 cells (two different strains). Although, as shown in Fig. 4, the part of these curves that correspond to low receptor occupancy are linear. Some levels of occupancy below saturation of the endocytic apparatus are far easier to achieve experimentally, it seems more reasonable to examine this aspect of the endocytic process. The experimental parameter $k_{10}$, which expresses the endocytic capacity of the $k_{10}$, seems more useful than estimates of individual parameters such as $k_{15}$ or $k_{10}$. It also conveys the appropriate impression that we can estimate a rate for the overall process without knowing the exact mechanism being regulated. Reversible estimates of individual endocytic parameters would require levels of receptor occupancy that are generally unattainable. Nevertheless, it seems clear that all of our experimental data is consistent with the Eq. 15 using very reasonable parameter estimates.
Quantitative analysis of the endocytic system involved in hormone-induced receptor internalization.

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