The Endocytotic Rate Constant

A CELLULAR PARAMETER FOR QUANTITATING RECEPTOR-MEDIATED ENDOCYTOSIS*

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The endocytotic rate constant \( k_e \) is a cellular constant which defines the probability of an occupied receptor being internalized in 1 min at \( 37 \) °C. We have derived an equation that expresses the relationship between \( k_e \) and the relative amount of surface-bound and internalized ligand as a function of time. This equation was used as the basis for a graphical form of analysis called the In/Sur plot. This plot directly yields \( k_e \) as well as the lag time that exists between ligand internalization and degradation. Accurate utilization of the In/Sur plot is dependent upon precise discrimination of surface-bound and internalized ligand, the existence of a lag time between ligand internalization and degradation, and a relatively constant amount of surface-bound ligand with respect to time. In studies utilizing \(^{125}\)I-labeled epidermal growth factor (EGF) and human fibroblasts, we found that all three of the above conditions could be satisfied using physiological temperatures and ligand concentration that spanned the mitogenic range for this cell type. There was approximately a 16% chance that an occupied EGF receptor would be internalized in 1 min at \( 37 \) °C. After internalization, there was a delay of at least 15 min prior to the initiation of EGF degradation. The \( k_e \) value for EGF receptors was constant independent of the total number of occupied cell surface receptors, indicating that receptors cluster to independent internalization sites rather than to each other. We also found that it was possible to measure the effects of environmental perturbations such as temperature and pH on both endocytosis and ligand binding, thus allowing one to quantitate the role of these processes in the overall interaction of ligands with target cells.

Receptor-mediated endocytosis is a process by which ligands that are specifically bound to cell surface receptors are rapidly partitioned into intracellular vesicles. Besides its classical role in the uptake of cellular metabolic precursors (such as low density lipoprotein \( 1 \) and vitellogenin \( 2 \)), receptor-mediated endocytosis also seems to be a universal occurrence in the interaction of polypeptide hormones with cells \( 3 \). Unfortunately, quantitative evaluation of receptor-mediated endocytosis is difficult since the interaction of polypeptide ligands with cells is a complex interplay between ligand binding, internalization, and degradation \( 4,5 \). Because of the apparent interdependent nature of these events, descriptions of endocytosis have been mainly restricted to the qualitative level \( 6-9 \).

We have recently presented a set of steady state equations that accurately describe the cellular binding, internalization, and degradation of polypeptide ligands under physiological conditions \( 5 \). A central concept in our steady state model is the endocytotic rate constant \( k_e \) which we defined as the probability of an occupied receptor being internalized in 1 min at \( 37 \) °C. The ligand-receptor \( k_e \) is important because its value can be used in conjunction with our steady state equations \( 5 \) to determine other cellular rate constants such as \( V \), the rate of insertion of new receptors into the cell surface; \( k_t \), the rate constant of internalization of empty receptors; and \( k_d \) (the ligand-receptor dissociation rate constant). Our previously described method for measuring \( k_e \) was dependent upon the existence of a cellular steady state. However, this steady state provision is somewhat restrictive since it is often desirable to quantify receptor-mediated endocytosis under initial conditions of cells approaching a steady state or after treatment with endocytotic modulators that could alter steady state conditions \( 5 \).

In the present paper, we define the theoretical and experimental conditions under which \( k_e \) can be accurately measured. We found that these conditions can be met not only at a steady state, but also as cells approach a steady state. These findings enabled us to develop refined methods for measuring \( k_e \) that are fast, accurate, and sensitive. Additionally, we describe a simple procedure for accurately measuring the lag time that exists between ligand binding and ligand degradation. These methods should facilitate studies on the role of these processes in the regulation of cellular activities by polypeptide hormones.

EXPERIMENTAL PROCEDURES

Reagents and Solutions—CHAPS \(^{1} \) was synthesized by the method of Hjelmeland \( 10 \) and was recrystallized 3 times from absolute methanol (also available from Polysciences, Inc.). Mouse EGF was isolated by the method of Savage and Cohen \( 11 \). All other chemicals were from Sigma. The EGF-binding solution was DV medium (Flow Laboratories) lacking bicarbonate but containing 20 mM Hepes, penicillin at 100 units/ml, streptomycin at 100 \( \mu \)g/ml, glutamine at 4 mM, and 1 mg/ml of BSA (RIA grade, Sigma). The pH was adjusted to 7.4 at 37 °C and the solution was sterilized by filtration. Cells were rinsed with a Hepes-buffered saline (HP saline) containing 0.1% polyvinylpyrrolidone \( (M_\theta = 40,000) \), 130 mM NaCl, 5 mM KCl, 0.5 mM MgCl\(_2\), 1 mM CaCl\(_2\), 20 mM Hepes, and NaOH to give a pH of 7.5.

Preparation of \(^{125}\)I-EGF—We found that \(^{125}\)I-EGF prepared by the standard chloramine-T procedure \( 12 \) is unsuitable for accurate studies on EGF binding and uptake. Chloramine-T-labeled EGF

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\(^{1}\) The abbreviations used are: CHAPS, 3[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DV medium, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; HF cells, human neonatal foreskin fibroblasts; In/Sur, the ratio between internalized ligand and cell surface-bound ligand; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin.
spontaneously cross-links to its receptor (13) which complicates the analysis of ligand binding. In addition, the chloramine-T procedure results in the apparent fragmentation of EGF, preventing quantitative analysis of EGF degradation (14). We thus developed a gentle method for the radiiodination of EGF that circumvents these problems. This method employs 1,4,6-triiodo-3,5-di-tyrosylglycyl-glycyl-glycine (Iodogen, Pierce Chemical Co.). Tubes coated with Iodogen were prepared by dissolving the Iodogen in chloroform to a concentration of 416 μg/ml and then placing 60-μl aliquots in pyrex culture tubes (10 × 75 mm) (25 μg/tube). The tubes were dried overnight under reduced pressure and could be stored at least a year in a vacuum desiccator at room temperature. For the iodination of EGF, the Iodogen-coated tube was rinsed with cold 100 mM Hepes buffer (pH 7.4) and placed on ice. 3 mCi of carrier-free 125I (Amersham) in a volume of 30 μl was added together with 20 μl of the 100 mM Hepes buffer. The EGF was then added (10 μg in 10 μl of 190 μM Hepes buffer) and after gentle mixing the tube was stood to stand on ice for 15 min. The reaction mixture was then transferred to a 1.5-ml microfuge tube and the Iodogen tube was rinsed with 940 μl of 75 mM NaCl, 20 mM Hepes (pH 7.4), 1 mM KL, and 0.1% BSA (RIA grade, Sigma). This rinse and the reaction mixture were pooled and dialyzed overnight against two changes of 75 mM NaCl, 20 mM Hepes (pH 7.4) at 6°C using Spectrapor; 3 tubing (M, cutoff = 3500). Recovery of 125I-EGF was virtually quantitative under these conditions. The 125I-EGF was used as a single band by native gel electrophoresis with <1% of the total radioactivity appearing as free iodine or partial breakdown products. Typical specific activities ranged between 230,000-280,000 cpmpg (~80% counting efficiency).

Cell Culture—Stock cultures of human fibroblasts from neonatal foreskins were prepared by a collagenase-dispase digestion technique.* The same stock (HF-SW8) was used in all of the studies presented here. Stock cultures were grown in DV medium containing 10% calf serum (Irvine Scientific) as previously described (5). Cultures were plated at a density of 1.5 × 10^4 cells/dish for use in experiments.

Analysis of 125I-EGF Binding—Unless otherwise noted, all binding measurements were performed on confluent cultures of HF cells in 60-mm dishes at 37°C. At least 4 h before appropriate times, duplicate 60-mm dishes were washed 3 times with 0.1% BSA (RIA grade, Sigma) cast in tubes (25 × 0.6 cm, inner diameter). The gel buffers were 0.062 M Tris, 0.06 M HCl for the stacking gel and 0.375 M Tris, 0.06 M HCl for the separating gel. The reservoir buffers were 40 mM Tris and 80 mM glycine. The gels were electrophoresed at 3 mA/gel at 4°C until the phenol red front was 0.3 cm from the bottom (4-5 h). The gels were frozen and cut into 0.2-cm sections and the radioactivity was measured in a γ counter. The radioactivity in the original sample was quantitatively recovered from the separating gel. Degradation was expressed as a percentage of the radioactivity loaded on the gel that migrated to the bottom of the gel as diiodothyrosine. The identities of the radioactive peaks on the gels were established as follows. The radioactivity in the gel slices was eluted overnight in 1 ml of water on a shaker platform. The eluate was then taken to dryness in a Savant Speed Concentrator and the residue dissolved in acetylated with 0.95% diiodothyrosine standards were visualized by ninhydrin. Relative Rf values for the standards in this system were EGF, 0.0; monodiodothyrosine, 0.43; diiodothyrosine, 0.54; iodide, 0.72; and iodine, 0.95.

RESULTS

Derivation of the Equations for Measuring k. —The binding of a ligand to its cell surface receptor is the first step in receptor-mediated endocytosis. The pool of occupied cell surface receptors can thus be considered the substrate of endocytosis. If we consider the case in which the internalization process is first order with respect to the occupied receptors, then

where T1 is the true value of internalized ligand, O1 is its observed value, TS is the true value of surface-bound ligand, OS is its observed value, and SS and IS are the fraction of spillover from the surface and interior of the cell, respectively. These values were set at 3.1 for SS and 0.06 for IS as discussed under "Results." T1 and TS were then initially set at the observed values and 20 iterations were used to approximate the final values. The corrected ratios of internal to surface-bound ligand were fit to Equation 5 under "Results" by the method of least squares. We have written a complete program for rapidly calculating values of k and surface-bound ligand from raw data in BASIC and machine languages for 600-based microcomputers. This program as prepared for a 48K Apple II Plus system is available on request.

Inhibition of Endocytosis by Phenylarsineoxide—PhAsO was made up as a 0.1 M stock solution in dimethylsulfoxide. Concentrations from 10^-3 to 10^-5 M were made in HP saline and added to 0°C. Confluent monolayers of cells were rinse with HP saline at 0°C and then treated with the PhAsO solutions at 0°C for 1 h. The cells were then rinsed twice with HP saline prior to performing binding at 37°C. Cells pretreated with 10^-3 M PhAsO for 1 h had an undetectable level of fluid phase endocytosis at 37°C and did not detectably secrete or internalize protease-nexin (also see Ref. 16).

Analysis of EGF Degradation—Confluent cultures of HF cells on 60-mm dishes were incubated with EGF in 1 ml of binding medium with 25 μg/ml of BSA for various lengths of time at 37°C. The incubations were terminated by adding 0.1 ml of 100 mM CHAPS, 20 mM EDTA, 20 mM PMPS (in a slurry). The PMPS was added from a 0.4 M ethanol stock just prior to use. The plates were placed on an ice bath for 2 min and the solution was then removed and placed in a microfuge tube together with 2 drops of glycerol. After mixing, insoluble debris was removed by centrifugation in a Beckman Microfuge for 1 min. The 125I-EGF and 125I-EGF degradation products in the solubilized samples were analyzed by a electrophoretic method based on the gel system of Ornstein (17) and Davis (18). The entire sample was loaded on the top of a native gel consisting of a 7-cm 12.5% acrylamide separating gel with 2% of the total acrylamide as N,N'-methylebisacrylamide (12.5% T, 2% C) and a 6-cm stacking gel containing 1.5% acrylamide with 3.25% of the acrylamide as N,N'-methylebisacrylamide (3.25% T, 20% C) cast in tubes (20 × 0.6 cm, inner diameter). The gel buffers were 0.062 M Tris, 0.06 M HCl for the stacking gel and 0.375 M Tris, 0.06 M HCl for the separating gel. The reservoir buffers were 40 mM Tris and 80 mM glycine. The gels were electrophoresed at 3 mA/gel at 4°C until the phenol red front was 0.3 cm from the bottom (4-5 h). The gels were frozen and cut into 0.2-cm sections and the radioactivity was measured in a γ counter. The radioactivity in the original sample was quantitatively recovered from the separating gel. Degradation was expressed as a percentage of the radioactivity loaded on the gel that migrated to the bottom of the gel as diiodothyrosine. The identities of the radioactive peaks on the gels were established as follows. The radioactivity in the gel slices was eluted overnight in 1 ml of water on a shaker platform. The eluate was then taken to dryness in a Savant Speed Concentrator and the residue dissolved in acetylated with 0.95% diiodothyrosine standards were visualized by ninhydrin. Relative Rf values for the standards in this system were EGF, 0.0; monodiodothyrosine, 0.43; diiodothyrosine, 0.54; iodide, 0.72; and iodine, 0.95.

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3 H. S. Wiley and D. A. Low, unpublished observation.
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\[
\frac{d[LR]}{dt} = k_e[LR]_i
\]

where \([LR]\) and \([LR]_i\) are the concentrations of the ligand-receptor complex at the surface of the cell and in the interior of the cell, respectively, and \(k_e\) is the endocytotic rate constant. This equation is valid only when there is no removal of ligand from the interior of the cell (i.e. no degradation). Rearranging Equation 1 yields

\[
\frac{d[LR]}{[LR]_i} = k_e \, dt.
\]

Now, if \(\frac{d[LR]}{dt} = 0\), that is, if the concentration of the ligand-receptor complex at the cell surface is approximately constant, then we can integrate the above equation as follows.

\[
\frac{1}{[LR]} \left[LR]_i \right]_{t_0}^{t} \, dt = k_e \left( t - t_0 \right).
\]

The solution of this integral is

\[
\frac{[LR] - [LR]_i}{[LR]} = k_e (t - t_0).
\]

If at the initial time \((t_0)\) the quantity of receptor complex inside the cell is zero \(([LR]_i = 0)\), then

\[
\frac{[LR]}{[LR]} = k_e \, t.
\]

This equation was derived previously using a different approach (5). However, it is clear from the above derivation that the validity of Equation 5 is not strictly dependent on steady state conditions. That is, the condition of \(\frac{d[LR]}{dt} = 0\) will give an exact solution to Equation 2, but the condition of \(\frac{d[LR]}{dt} \approx \frac{d[LR]}{[LR]_i} \approx 0\) will give a close approximation of \(k_e\). Of course, the condition that \([LR]_i\) is a constant exists at steady state by definition, but the approximate condition can theoretically be met if the time period of measurement \((t - t_0)\), see Equation 4) is sufficiently short and if \(\frac{d[LR]}{dt} \approx \frac{d[LR]}{[LR]_i}/dt\).

Equation 5 is that of a straight line and predicts that plotting the ratio of internalized ligand to surface-bound ligand as a function of time will yield a straight line with a slope of \(k_e\). However, in order for this equation to be valid, not only must the amount of surface-bound ligand approximate a constant but there must also be no degradation or loss of internalized ligand during the time course of measurement.

The above derivations show that \(k_e\) can be measured if three conditions are satisfied: 1) The internalized ligand and surface-bound ligand can be quantitatively discriminated from each other. 2) There is no degradation or removal of the internalized ligand during the time course of measurement. 3) The quantity of ligand bound to cell surface receptors approximates a constant during the time course of measurement. Therefore, we investigated the conditions under which the above three criteria can be met. These experiments, presented below, permitted a test of the validity of Equation 5 for directly measuring \(k_e\). They utilized \(^{125}\text{I}-\text{EGF}\) and HF cells, a system that is well suited for investigating receptor-mediated endocytosis (4, 6).

Discrimination of Surface-bound and Internalized Ligand—It has been reported that \(^{125}\text{I}-\text{EGF}\) bound to the cell surface can be selectively removed by an acidic acid treatment at 0°C. Haigler et al. (15) reported that this technique is capable of removing >90% of the \(^{125}\text{I}-\text{EGF}\) associated with the surface of mouse 3T3 cells while removing <5% of the internalized ligand. To verify the applicability of this technique to HF cells, we evaluated removal of \(^{125}\text{I}-\text{EGF}\) associated with the cell under conditions in which the ligand was either totally localized at the cell surface or totally localized inside the cell. To localize \(^{125}\text{I}-\text{EGF}\) entirely to the cell surface, HF cells were incubated with \(^{125}\text{I}-\text{EGF}\) at 0°C. Cells will not internalize ligands at this temperature (4, 9, 15, 21, 29). After removing the unbound \(^{125}\text{I}-\text{EGF}\), we treated the cells with the acetic acid solution for various times and measured the percentage of total ligand remaining with the cells. As presented in Fig. 1, the 4-min acid treatment removed ~90% but not all of the surface-associated ligand. Additional rinsings with the acid solution did not remove the resistant ligand. This inability to remove all of the cell surface-bound ligand may have been due to performing the initial binding at 0°C. To determine the quantity of surface-associated ligand at physiological binding temperatures that was resistant to acid removal, we used PhAsO to block internalization. PhAsO is an extremely potent inhibitor of endocytosis in both amphibian oocytes (23) and mammalian cells (16); a brief pretreatment at 0°C with 10^-4 M PhAsO is sufficient to irreversibly block endocytosis. Cells were thus pretreated with various concentrations of PhAsO at 0°C for 1 h and the inhibitor was subsequently removed prior to performing ligand binding at 37°C. We then tested the ability of the acid treatment to remove cell-associated ligand. The results presented in Fig. 2 showed that at increasing concentrations of PhAsO, a greater percentage of the total cell-associated ligand became accessible to acid removal. However, even at concentrations of PhAsO that totally block endocytosis, ~10% of the radioactivity associated with the cell surface could not be removed by acid treatment. This confirms the above results obtained by binding at 0°C (Fig. 1).

To test the possibility that the acid treatment might remove internalized ligand, we first incubated HF cells with \(^{125}\text{I}-\text{EGF}\) for 6 h to produce a steady state pool of internalized ligand (5). The cells were then switched to medium containing unlabeled EGF to remove surface-associated \(^{125}\text{I}-\text{EGF}\) labeled ligand by dissociation and/or internalization. The time course of the loss of “surface” ligand and “internal” ligand as measured by the acid stripping technique was then determined and is presented in Fig. 3. The loss of the internal ligand with respect to time followed an apparent first order decay curve as previously noted (5). The apparent surface-associated ligand was lost very rapidly at first but then the rate of loss decreased and paralleled the loss of internalized ligand. The close similarity between the half-life of the internal ligand and the persistent surface ligand (46 versus 43 min) indicates that this component of the surface ligand was internalized ligand that was removed by the acid treatment. This component comprised approximately 5% of the total internal ligand. Thus, the finding of Haigler et al. (15) that the acid stripping technique removes >90% of the surface ligand while only removing ~5% of the internalized ligand with 3T3 cells applies also to HF cells.

To permit a more quantitative discrimination of surface-bound and internalized ligand, we designed a computer program to correct the observed values of surface and internalized ligand using the above determined spillover values. We used this program to generate the “correction curve” presented in Fig. 4. This figure illustrates the difference between observed and actual In/Sur ligand ratios and can also be used to correct observed values. However, there is less than a 10% difference between the observed and actual values up to an In/Sur ratio of ~3. Thus, the most accurate In/Sur ratios will be obtained within this range.

Determination of Lag Time for EGF Degradation—Several research groups have suggested that there is a lag time
iodotyrosine peak and accounted for about 15% of the total EGF as degradation products. We have found that this electrophoretic technique is capable of detecting -0.5% of the total free iodide. TLC analysis. The peak at slices 21-24 (Fig. 5B) was identified as 80% mono[125I]iodotyrosine, 10% di[125I]iodotyrosine, and 10% unidentified degradation products by TLC analysis. The peak at slices 31-34 was identified as 75% free iodide, 20% mono[125I]iodotyrosine and 5% unidentified degradation products. The increase in the radioactivity of the gel front was parallel to that of the main mono- and di[125I]iodotyrosine peak and accounted for about 15% of the total EGF degradation products. We have found that this electrophoretic technique is capable of detecting ~0.5% of the total EGF as degradation products.

between EGF internalization and degradation (9, 24-26). However, the techniques used to determine this point (electron microscopy, TCA precipitation, or antibody precipitation) are not quantitative (9, 14) and are thus not suitable for detecting very small amounts of degradation products that might be initially produced by cells. Since the derivations presented above showed that measurements of \( k_1 \) depend on no degradation of internalized ligand during the course of the measurement, it was critical to determine if such a lag time really exists. We thus developed a sensitive electrophoretic technique for measuring EGF degradation. Cells are incubated in \( ^{125}\text{I}-\text{IGF} \)-containing medium for different times and the incubation is terminated by addition of the detergent CHAPS and a protease inhibitor (PMSF) to dissolve the cells and prevent further degradation (see "Experimental Procedures"). This step releases internalized EGF and intracellular degradation products. The entire sample (medium plus cells) is then rinsed and warmed to 37 °C. Binding was performed with 5 ng/ml of \( ^{125}\text{I}-\text{EGF} \) for 15 min at 37 °C and the per cent of specifically bound EGF that could not be removed by the acetic acid treatment was determined as outlined under "Experimental Procedures." At 10⁻⁴ M PhAsO, fluid phase endocytosis was totally blocked as measured by \( ^{3}\text{H}\)-polivinylpyrrolidone uptake.

Fig. 3 (right). Time course of loss of internalized and surface-bound EGF. Cells were brought to a steady state with 5 ng/ml of \( ^{125}\text{I}-\text{EGF} \) for 6 h, and then switched to medium containing 5 ng/ml of unlabeled EGF. At the indicated times, the amount of radioactivity that was removed by acid treatment (O—O) or resistant to acid treatment (■—■) was determined as outlined under "Experimental Procedures." The lines through the data points were determined by least squares analysis of the time points at 10 min and beyond.

To determine the lag time between the initial interaction of EGF with HF cells and the initiation of its degradation, we incubated cells with 1 ng/ml of \( ^{125}\text{I}-\text{EGF} \) at 37 °C and at different times measured the per cent of total radioactivity that was present as mono- and di[125I]tyrosines. The sensitivity of our assay for EGF degradation was further increased by incubating the cells in a small volume of medium (1 ml/60-mm dish). As presented in Fig. 6, there was no detectable degradation during the first 15 min of incubation, but, thereafter, there was a significant generation of \([125\text{I}]\)tyrosines by the cells. The generation of these degradation products were completely blocked by the addition of 1 µg/ml of unlabeled EGF (results not shown). Our estimated degradation lag time (15-20 min) is significantly shorter than previously reported values (30-60 min, see Refs. 25, 26), and probably reflects the increased sensitivity of the electrophoretic assay.

Since there is at least a 15-min lag time prior to the onset of EGF degradation in HF cells, binding data plotted according to Equation 5 should produce a straight line for at least the first 15 min after adding EGF to the cells. This was tested by adding 1 ng/ml of \( ^{125}\text{I}-\text{EGF} \) to the cells and measuring the In/Sur ratios at 3 min intervals. The result of this experiment is shown in Fig. 7 and confirmed our expectations. The data points fit a straight line (correlation coefficient >0.99) for the first 15 min after ligand addition. Thereafter, the curve breaks sharply. The break in the curve corresponds to the time at which degradation products of EGF were first observed (Fig. 6).

We found that the lag time prior to the onset of EGF degradation is a characteristic of the particular cell type and the culture conditions used. Experiments performed in the presence of serum resulted in significantly longer lag times (20-30 min), while experiments performed in HP saline or at...
higher temperatures (~41 °C) resulted in shorter lag times (~12 min). Secondary mouse embryo cells in serum-free medium had lag time of approximately 8 min, while Chinese hamster embryonic fibroblasts displayed lag times (in 5% serum) of 6-11 min depending on the clonal line used. However, in every cell type examined, there was always a finite lag time observed. The observational "window" that can be used for kₗ determinations has to be experimentally determined for any particular type of cells and binding conditions.

**Kₗ as a Function of Ligand Concentration**—In order for the initial slope of the In/Sur plot presented in Fig. 7 to be an accurate measure of kₗ, the change in the amount of surface-associated ligand with respect to time must be small. This situation should exist for EGF at very low ligand concentrations since the loss of surface receptors with time by internalization is significant only at high EGF concentrations (5). Thus, we investigated the effect of EGF concentration on apparent kₗ values to see if we could obtain a constant value as long as the amount of surface-bound ligand remained constant. The results of this experiment are presented in Fig. 8. Shown are the measured kₗ values at the indicated EGF concentrations together with the time course of surface-ligand binding for three of the EGF concentrations. At the lowest EGF concentration tested (0.03 ng/ml), the quantity of surface-associated ligand remained constant during the entire 15 min assay and the In/Sur plot yielded a straight line with a correlation coefficient of >0.99 and a slope of 0.166 min⁻¹. The average value of kₗ for EGF concentrations up to 1 ng/ml was 0.157 min⁻¹ with a standard deviation of 0.008 min⁻¹. Within this concentration range, the amount of EGF associated with the cell surface with respect to time was approximately con-
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Fig. 7. In/Sur analysis of 125I-EGF and HF Cells. Confluent cultures of HF cells were incubated for the indicated times at 37 °C with 1 ng/ml of 125I-EGF. The ratio of the radioactivity associated with the interior to the surface of the cells (In/Sur ratio) was then determined as outlined under "Experimental Procedures." The line through the first five points had a correlation coefficient of >0.99. The slope of this line yielded a $k_r$ value of 0.153 min⁻¹.

Fig. 8. Effect of EGF concentration on measured values of $k_r$, and on occupied receptor clearance rates from the surface of HF cells. Confluent cultures of HF cells were incubated with the indicated concentrations of 125I-EGF, and $k_r$ was measured as described under "Experimental Procedures." The lower graph presents the observed $k_r$ values (▲—▲) as a function of 125I-EGF concentration. The upper three graphs show the time course of association of 125I-EGF with the surface of the cells (●—●). These data were derived during measurements of the $k_r$ values at the EGF concentrations indicated. Note that even though the amount of surface-associated radioactivity increases dramatically at 1.0 ng/ml of EGF compared to 0.03 ng/ml of EGF, values of $k_r$ remain constant as long as the surface-associated radioactivity remains constant.

Fig. 9. Effect of temperature on $k_r$ and cell surface binding of 125I-EGF to HF cells. The cells in 35-mm dishes were incubated at the indicated temperatures with 1 ng/ml of 125I-EGF and the values of $k_r$ were determined as outlined under "Experimental Procedures." Shown are the values of $k_r$ (●—●) together with the average amount of radioactivity specifically associated with the surface of the cells (△—△) during the measurement of $k_r$. The error bars are the standard deviations of the means (total of 10 plates for each point). The above experiment demonstrated that $k_r$ could be used as a cellular parameter for investigating the effect of environmental perturbations on receptor-mediated endocytosis exclusive of their effect on net ligand binding. To further verify the independence of $k_r$ from net ligand binding, we investigated the effect of pH on these two cellular parameters. As shown in Fig. 10, both $k_r$ and average surface binding had an optimal
The interaction of polypeptide ligands with cells is a complex interplay between ligand binding, internalization, and degradation. A quantitative evaluation of the cellular parameters involved in these interactions is necessary to understand cellular modulation of ligand-receptor processing. Previous investigators have attempted to quantitate receptor-mediated endocytosis by measuring the net rate of ligand internalization as a function of the extracellular ligand concentration. However, occupied cell surface receptors are the true substrates of endocytosis, not free ligand. Additionally, the net rate of ligand internalization is influenced by a number of factors such as receptor affinity and receptor number. Thus, a change in the internalization rate does not necessarily indicate the cellular level at which the change has occurred.

A much more useful measure of the ability of cells to internalize a ligand-receptor complex is $k_e$. This constant is independent of receptor affinity or number and, thus, is a more accurate measurement of internalization per se. We have developed the In/Sur plot as a means of accurately measuring $k_e$ since this method expresses internalization as a function of occupied surface receptors and not as a function of ligand concentration. Indeed, we found that $k_e$ was essentially independent of the concentration of ligand in the medium (see Fig. 8) and behaved as a first order rate constant. We do not mean to imply that a single molecular mechanism is responsible for the transfer of an occupied receptor to an intracellular compartment, but simply that there is only a single rate limiting step in this process. Thus, $k_e$ can be used to quantitate the regulation of this cellular process.

Three conditions must be met to measure the $k_e$ of a cell for a particular ligand. One must be able to quantitatively discriminate between the surface-bound and internalized ligand. There must be a lag time between internalization and degradation. Finally the quantity of surface-bound ligand must remain approximately constant during the experimental measurement. The acid-stripping technique of Haigler et al. (15) enables one to easily discriminate between surface-bound and internalized EGF. Unfortunately, the technique is not strictly quantitative. We thus developed a computer program to compensate for the slight spillover observed between the surface and internal compartments of the cell and this allowed us to accurately measure the relative amount of ligand associated with the surface and interior of the cell. However, the error in $k_e$ introduced by ignoring this spillover is small at short time intervals and results in about 10% underestimation of the value of $k_e$.

We confirmed the observation of other workers that there is a lag time between EGF binding and degradation (9, 24–26). A lag time for degradation seems to occur for all ligands internalized by receptor-mediated endocytosis (7, 24, 30, 31) and may reflect the existence of a privileged "receptosome" compartment indicated by Willingham and Pasan (32), as well as by other investigators (7, 33). However, using the very sensitive electrophoretic technique described in this paper, we found that the time between the initial binding of EGF and the initiation of its degradation is considerably shorter than previously reported. Our plots of In/Sur versus time revealed the time of the initiation of degradation by a sharp break in the plot (see Fig. 7). We found that under standardized conditions, the degradation lag time can be quantitated with an accuracy of better than 1 min. Thus, In/Sur plots can also be a very useful tool for investigating the molecular mechanisms responsible for the degradation lag time.

The In/Sur plot allows one to quantitatively evaluate separately the relative contributions of ligand binding and internalization to the overall interaction of ligands with cells. As demonstrated in Figs. 9 and 10, this technique was able to discriminate the relative effect of temperature and pH on cell surface binding and $k_e$. Note that the temperature optimum for $k_e$ is ~36 °C, which is physiological for these cells, while the temperature optimum for receptor binding is ~29 °C. Therefore, binding studies performed at lower temperatures (20–30 °C) will yield results indicating a much larger relative role for cell surface binding than what actually occurs at physiological temperatures.

The In/Sur plot not only allows accurate quantitation of receptor-mediated endocytosis in overall ligand-cell interactions, but also allows one to set constraints on any molecular model of the regulation of receptor-mediated endocytosis. For example, the observation that $k_e$ acts as a first order rate constant indicates that occupied receptors cluster at independent sites on the cell surface and not to each other. It has been shown that the increase in receptor internalization seen upon addition of EGF to clusters is accompanied by a parallel increase in the clustering of these receptors (9, 24). If receptors clustered to each other, then the probability of "hitting" or clustering to another occupied receptor would increase with increasing receptor occupancy and this would be expected to affect the rate of their internalization. However, no such ligand concentration effects were observed (see Fig. 8), indicating the possible existence of clustering sites extrinsic to the occupied receptor. This hypothesis is consistent with the observations that most (if not all) occupied receptors are clustered together at the cell surface and internalized in the same endocytic vesicles (6). In like manner, the In/Sur plot can be used to investigate the effect of ions and specific proteins on specific receptor-mediated endocytosis. For example, calcium has been postulated to be important in the
process of ligand internalization (21). However, we found that the presence or absence of calcium or EGTA has not detectable effect on the $k_e$ for EGF (results not shown), indicating that its previously reported effects on ligand internalization resulted from effects on other events such as ligand binding (21). However, calf serum (5%) lowered $k_e$ by about 20% compared to serum-free medium indicating that this process can be modulated by serum and perhaps other regulatory agents. Thus, the use of the In/Sur plot, together with a number of labeled ligands, should enable one to systematically investigate the factors regulating receptor-mediated endocytosis.

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