Anomalous Binding of Epidermal Growth Factor to A431 Cells Is Due to the Effect of High Receptor Densities and a Saturable Endocytic System

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Abstract. This study was conducted to determine how extraordinarily high numbers of epidermal growth factor receptors (EGF-R) affected the binding and internalization of EGF in the transformed cell line A431. I found that at low EGF concentrations, the kinetics of binding behaved as a nonsaturable, first-order process showing no evidence of multiple-affinity classes of receptors. However, EGF dissociation rates were strongly dependent on the degree of receptor occupancy in both intact cells and isolated membranes. This occupancy-dependent dissociation appears to be due to diffusion-limited binding. EGF-induced receptor internalization was rapid and first order when the absolute number of occupied receptors was below $4 \times 10^3$ min$^{-1}$. However, at higher occupancies the specific internalization rate progressively declined to a final limiting value of 20% normal. The saturation of EGF-R endocytosis was specific since internalization of transferrin receptors was not affected by high concentrations of either transferrin or EGF. Saturation of EGF-R endocytosis probably involves a specific component of the endocytic pathway since fluid phase endocytosis increased coordinately with EGF-R occupancy. I conclude that there are several aspects of EGF-R dynamics on A431 cells are neither similar to the behavior of EGF-R in other cell types nor similar to the reported behavior of other hormone receptors. Although A431 cells have an extraordinary number of EGF-R, they do not seem to have corresponding levels of at least two other crucial cell surface components: one that mediates EGF-induced rapid receptor internalization and one that attenuates EGF-induced membrane responses. These factors, in addition to the presence of diffusion-limited binding at low EGF concentrations, are probably responsible for the appearance of multiple-affinity classes of receptors in this cell type.

The epidermal growth factor receptor (EGF-R) is one of the best-characterized hormone/growth factor receptors. It has been purified to homogeneity (Cohen, 1983), its gene has been cloned (Lin et al., 1984), and its complete primary sequence has been determined (Ullrich et al., 1984). Much is known about its intrinsic protein kinase activity (Cohen et al., 1980), autophosphorylation (Weber et al., 1984), and cellular substrates (Haigler et al., 1987). A primary reason for this rapid progress has been the introduction of the A431 cell line as a model system (Haigler et al., 1978). This cell line possesses extraordinary numbers of receptors, estimated within the range of 2–4 million per cell (Haigler et al., 1979a; Krupp et al., 1982; Gamou et al., 1984). The high level of EGF-R in A431 cells has not only facilitated investigations on their biochemistry and structure, but it has also aided investigations on their surface dynamics and route of internalization. By using EGF conjugated to ferritin and fluorescent dyes, Haigler and colleagues traced the internalization pathway in A431 cells (Haigler et al., 1978; Haigler et al., 1979a; McKanna et al., 1979). Schlessinger and co-workers used both fluorescent and phosphorescent probes to investigate receptor dynamics in that cell type (Zidovetzki et al., 1981; Hillman and Schlessinger, 1982). This and other data have been used to construct a general model of ligand-induced receptor-mediated endocytosis that includes the following features. The EGF-R is initially diffusely distributed at the cell surface. After EGF binds, the occupied receptors cluster to each other. These clusters localize over coated pits on the cell surface which then internalize the complexes (Haigler et al., 1979a; McKanna et al., 1979; Zidovetzki et al., 1981; Willingham et al., 1983). This model of ligand-induced clustering followed by internalization has been proposed for a variety of other cell and hormone receptor types (Pastan and Willingham, 1981; Anderson and Kaplan, 1983; Willingham et al., 1983). However, the relatively low number of receptors found on the surface of most cells has made it difficult to test.

Although A431 cells are an excellent system in which to investigate the biochemistry of the EGF-R, they are less than optimal for studying the regulation of EGF-induced cellular...
responses. The usual consequence of adding EGF to A431 cells is an acute membrane ruffling response followed by cell death (Haigler et al., 1979b; Gill and Lazar, 1981; Barnes, 1982), presumably caused by an overload of crucial metabolic networks (Buss et al., 1984). Thus most investigators working on EGF-induced cellular responses have used either human fibroblasts (Carpenter and Cohen, 1976a;b; Knauer et al., 1984) or mouse 3T3 cells (Aharonov et al., 1978) as their model system.

My colleagues and I have been developing a detailed, quantitative model of the regulation of receptor/membrane dynamics by EGF over the last several years (Wiley and Cunningham, 1981, 1982a; Knauer et al., 1984; Wiley and Kaplan, 1984). We noticed that the behavior of the EGF-R on human fibroblasts did not correspond to that reported for A431 cells. For example, we could find no evidence that a receptor–receptor clustering step was required for EGF-induced internalization in fibroblasts (Wiley and Cunningham, 1982a). Likewise, Carpenter et al. (1982) found no evidence for EGF-induced clustering of receptors using morphological analysis of EGF-R distribution in human fibroblasts. Instead, their data were consistent with EGF-R being preclustered in coated pits. Since the dynamic behavior of a wide variety of other receptor systems appears similar to that of the EGF-R in human fibroblasts (Anderson and Kaplan, 1983; Wiley, 1985), it seemed possible that certain aspects of the behavior of the EGF-R in A431 cells were the exception and not the rule. I therefore decided to investigate the dynamics of the EGF-R in A431 cells with the aim of identifying those aspects that are similar to other cells and those that are different.

In this article, I demonstrate that, at low concentrations of EGF, binding behaves as a diffusion-limited process. In addition, A431 cells are unable to efficiently internalize high levels of occupied EGF-R apparently because of a relatively low level of a specific component that mediates EGF-induced receptor internalization.

Materials and Methods

General

Mouse EGF was purified from submaxillary glands according to the method of Savage and Cohen (1972). The material gave a single band upon native gel electrophoresis (Wiley and Cunningham, 1982a). Human derrtic transferrin (TF) (98–99% electrophoretic purity) was purchased from Calbiochem-Behring Corp. (San Diego, CA) and was iron-loaded as described (Ward et al., 1982). Both EGF and TF were iodinated by the Iodo-Gen procedure as described (Wiley and Cunningham, 1982a) and free 125I was separated from the radiolabeled ligands by passing the mixtures over a 0.8 × 20-cm column of Sephadex G-10 equilibrated with PBS. The specific activity of the 125I-labeled EGF was generally between 760 and 1,700 cpm/mmol whereas that of the 125I-labeled TF was between 200 and 400 cpm/mmol.

The A431 cell line was obtained from Dr. Harry Haigler (University of California, Irvine) and grown as stocks in 100-mm dishes using DME (Flow Laboratories, McLean, VA) supplemented with 10% FCS (HyClone Laboratories, Logan, UT). Approximately 2 × 105 cells were seeded into 35-mm plates and grown to a final density of 1 × 105 cells per dish for experiments. The human foreskin fibroblasts used in this study were strain SW-8 prepared and cultured as described (Wiley and Cunningham, 1981, 1982a).

Plasma membranes from A431 cells were prepared by hypotonic shock of cells grown in roller bottles as described by Cohen et al. (1982). The final membrane preparation had a protein content of 7 mg/ml and was stored frozen at −70°C until use. Anti-EGF antiserum was prepared in rabbits by conjugating EGF to an ovalbumin carrier using glutaraldehyde as described (Moses et al., 1980). The fluid-phase marker 125I-polyvinylpyrrolidone (PVP) was prepared by the UV-catalyzed oxidative reaction as previously described (Wiley and McKinley, 1987). The specific activities were generally around 1 × 105 cpm/ml.

Receptor Binding Studies

Cells grown to confluence in 35-mm dishes were changed to serum-free medium 18 h before experiments (Knauer et al., 1984). To measure the initial association kinetics of 125I-EGF, cells were transferred to a 37°C tabletop binding incubator where the cells were kept on a stainless steel platform in a water bath. The addition of 125I-EGF as well as the rinsing of the cells with ice-cold saline was performed using the semiautomatic apparatus previously described (Wiley and Cunningham, 1982a). The temperature limits for all kinetic experiments was 35.5–37°C and the temporal resolution was within 10 s. Nonspecific binding was measured in the presence of 5–10 μg/ml unlabeled EGF. Cell number was determined with a Coulter counter (Coulter Electronics Inc., Hialeah, FL). The relative amounts of ligand associated with the surface and interior of the cells was determined using the acid-stripping technique previously described (Wiley and Cunningham, 1982a). All data were corrected for nonspecific binding and inside/surface spillover. The values of both the association and dissociation rate constants of the EGF-R were determined by curve fitting as previously described (Knauer et al., 1984). The value of the specific internalization rate of either the EGF or Tf receptors (kₐ) was determined by the internalization plot method (Opresko and Wiley, 1987a,b). The integral of surface binding that this method requires was determined by numeric integration of the surface data. Templates for the correction of all data and the automatic calculation of integral surface binding, average receptor occupancy, and kₐ were created for both SuperCalc 3a (Apple Ile computers) and for Microsoft Excel (Apple Macintosh computers). These templates or the spreadsheet formulas used to create them are available on request.

Results

Initial Binding Kinetics of EGF to A431 cells

As an initial experiment, I incubated confluent monolayers of A431 cells with EGF concentrations ranging from 1.7 × 10⁻¹¹ to 4.1 × 10⁻⁹ M 125I-EGF since these concentrations span the range used to discriminate between "high" and "low" affinity receptors (Kawamoto et al., 1983; Rees et al., 1984; Chatelier et al., 1986). The amount of ligand internalized and on the cell surface was then measured as a function of time. Shown in Fig. 1 is the initial kinetics of EGF binding to the surface of these cells at 37°C. The data is plotted as the relative binding (total binding divided by the ligand concentration) to facilitate comparisons. The first 2 min of binding was very similar at all ligand concentrations. Thereafter binding increased linearly at a rate that was dependent on ligand concentration. Surface binding at lower ligand concentrations was very similar to that obtained with 1.7 × 10⁻¹⁰ M ligand (data not shown). By 10 min, the total number of receptors lost by a combination of occupancy and ligand-induced internalization was 3.4 × 10⁵, 1.6 × 10⁶, and 6.6 × 10⁶ receptors per cell for 1.7 × 10⁻¹⁰, 8.3 × 10⁻¹⁰, and 4.1 × 10⁻⁹ M EGF, respectively. Paradoxically, the relative surface binding of EGF was highest for those cells treated with the greatest concentration of ligand and thus those that had lost the greatest number of receptors. The initial "apparent" second-order rate constant of EGF association ranged between 3.1 and 3.6 × 10⁶ M⁻¹s⁻¹ for the different EGF concentrations. These values are ~100-fold less than that reported for the EGF-R in other cell types (Hock and Hollenberg, 1980; Knauer et al., 1984).

The kinetics of EGF binding to the surface of A431 cells
Confluent monolayers of cells were incubated with \(^{125}\text{I}-\text{EGF}\) at concentrations of \(1.7 \times 10^{-10} \text{ M (o), 8.3} \times \!10^{-10} \text{ M (r), or 4.1} \times \!10^{-9} \text{ M (e)}\) and at the indicated times the amount of ligand associated with the cell surface was determined by acid stripping as described in Materials and Methods. The scales for the different curves were adjusted to factor out the relative ligand concentration as indicated along the y-axis.

Figure 1. Kinetics of surface binding of \(^{125}\text{I}-\text{EGF}\) in A431 cells. Confluent monolayers of cells were incubated with \(^{125}\text{I}-\text{EGF}\) at concentrations of \(1.7 \times 10^{-10} \text{ M (o), 8.3} \times \!10^{-10} \text{ M (r), or 4.1} \times \!10^{-9} \text{ M (e)}\) and at the indicated times the amount of ligand associated with the cell surface was determined by acid stripping as described in Materials and Methods. The scales for the different curves were adjusted to factor out the relative ligand concentration as indicated along the y-axis.

Figure 2. Kinetics of surface binding of \(^{125}\text{I}-\text{EGF}\) in normal human fibroblasts. Confluent monolayers of cells were incubated with \(^{125}\text{I}-\text{EGF}\) at concentrations of \(1.3 \times 10^{-10} \text{ M (o), 5.3} \times \!10^{-10} \text{ M (r), or 2.1} \times \!10^{-9} \text{ M (e)}\) and at the indicated times the amount of ligand associated with the cell surface was determined by acid stripping as described in Materials and Methods. The scales for the different curves were adjusted to factor out the relative ligand concentration as indicated along the y-axis.
the binding of EGF to A431 cells behaves as a diffusion-limited process.

Although the above results can explain the anomalous linear binding isotherms of 125I-EGF in A431 cells, it does not explain the higher relative surface binding observed at higher ligand concentrations (Fig. 1). One possible explanation was that the cells were internalizing EGF less effectively at higher receptor occupancies. To more accurately assess this phenomenon, I incubated cells with either $1.7 \times 10^{-11}$ or $1.7 \times 10^{-9} \text{ M} 125\text{I}-\text{EGF}$ and then determined the relative amount of internalization by acid stripping. The data were then used to generate the internalization plot shown in Fig. 4. In an internalization plot, the slope of the curve at any point is proportional to $k_e$ (the specific internalization rate of the receptor; Opresko and Wiley, 1987a, 1987b). As shown in Fig. 4, the slope of the plot at $1.7 \times 10^{-9} \text{ M} 125\text{I}-\text{EGF}$ was significantly less than at the lower ligand concentration. This is in marked contrast to most other cell types in which the specific internalization rate of the receptor is a constant at all receptor occupancies (Wiley, 1985). Since higher specific internalization rates increase the apparent “affinity” of a receptor at 37°C, the presence of “high” and “low” affinity receptors in this cell type at physiological temperatures could at least be partially explained by differences in $k_e$ at the two ligand concentrations (Gex-Fabry and DeLisi, 1984; Wiley, 1985).

**The Specific Internalization Rate of the EGF Receptor Decreases as a Function of Receptor Occupancy**

To further explore the relationship between receptor occupancy and internalization, I performed binding studies on A431 cells using $125\text{I}-\text{EGF}$ concentrations ranging from $8.3 \times 10^{-12}$ to $1.6 \times 10^{-8} \text{ M}$. The data obtained were converted to internalization plots to find the value of $k_e$. These results were then plotted as a function of the average number of occupied receptors on the cell surface during the 10-min time period of the experiment (Fig. 5). For comparison, Fig. 5 also includes the results obtained with normal human fibroblasts. The internalization data from A431 cells describes a biphasic curve in which the specific internalization rate of the EGF-R is initially high, but then decreases with increasing occupancies. A limiting value of $k_e$ (0.04 min⁻¹) was reached at an average occupancy of $\sim 5 \times 10^7$ receptors.
Mechanisms for Saturable Internalization in A431 cells

There are at least three hypotheses that could explain the inhibition of EGF-R internalization at high receptor occupancies. First, there could be two classes of EGF receptors. The high affinity receptors would be rapidly internalized while the low affinity ones would be internalized more slowly. Second, high levels of occupied receptors on the cell surface could somehow compromise the ability of the cells to internalize coated pits. Third, occupied EGF-R could be competing for some limiting component that is required for their "induced" internalization. If the concentration of this component was less than that of the entire complement of surface EGF-R, then internalization would only be efficient when the level of receptor occupancy was below that of the rate-limiting component. The following experiments were designed to determine which of these hypotheses was the most likely to be correct.

If there are two different affinity classes of EGF receptors, then one could explain the occupancy-dependent internalization rates of the EGF-R by simply postulating that the high affinity receptors are internalized efficiently while the low affinity receptors are internalized slowly. Thus at low ligand concentrations, the high affinity/rapidly internalized receptors would be preferentially occupied and internalized. To test this hypothesis, I first incubated A431 cells with a very low ligand concentration (1.6 × 10^{-11} M) for 5 min to occupy some putative high affinity receptors (Kawamoto et al., 1983; Chatelier et al., 1986). The cells were then rinsed and incubated an additional 5 min in either the presence or absence of a high concentration of ligand (8 × 10^{-7} M). If there were two classes of receptors, then occupying the low affinity receptors with unlabeled ligand should not affect the internalization of the previously occupied high affinity ones. However, the data from this experiment (Fig. 6) show that this is not the case. When the cells were pulsed with a low concentration of 125I-EGF and then simply chased with the same concentration of ligand, the surface-associated ligand was rapidly internalized. Note that there is no apparent dissociation of 125I-EGF from the cells since all of the 125I-EGF lost from the cell surface appeared inside the cells. In contrast, when the cells were chased in the presence of a high concentration of unlabeled EGF, internalization of the previously bound ligand was essentially blocked and the 125I-EGF rapidly dissociated back into the medium (as was also the case with isolated membranes; Fig. 3). This experiment illustrates two important aspects of EGF-R dynamics in A431 cells. First, occupying large numbers of EGF-R interferes with the internalization of previously occupied receptors. Second, the apparent rate of dissociation of 125I-EGF from the surface of A431 cells is highly dependent on the density of unoccupied receptors.

I next tested the hypothesis that occupying large numbers of receptors on the surface of A431 cells could compromise the ability of the cells to either internalize or to "see" additional occupied receptors. In this scenario, large numbers of activated receptors could paralyze the cell and thus inhibit processes crucial to coated pit internalization. To test this hypothesis, I determined the relationship between receptor occupancy and the rate of fluid-phase endocytosis. It has been established that one of the most rapid cellular responses to EGF binding is an increase in the net rate of endocytosis (Haigler et al., 1979b; Wiley and Cunningham, 1982b). This most likely reflects compensatory endocytosis that oc-
EGF-stimulated fluid phase endocytosis is proportional to receptor occupancy at all concentrations of EGF. Confluent monolayers of A431 cells were incubated with \( ^{125}\text{I}-\text{PVP} \) and unlabelled EGF concentrations ranging from \( 1.7 \times 10^{-11} \) to \( 1.7 \times 10^{-8} \) M. The amount of \( ^{125}\text{I}-\text{PVP} \) internalized in a 15-min period was determined as previously described (Wiley and Cunningham, 1982) and corrected for cell number. A parallel group of cells was incubated with identical concentrations of \( ^{125}\text{I}-\text{EGF} \) and the average amount of ligand associated with the cells for 15 min (both surface-associated and internalized) was directly determined. These data were used to calculate the number of occupied EGF-R present during the fluid uptake experiments. All points are the average of duplicate determinations.

EGF Receptors Specifically Interfere with Each Other for Internalization

Although the above experiments suggested that there was interference occurring between EGF-R at high occupancies, they did not provide information regarding the mechanism of such a process. It seemed possible that there simply were not enough coated pits to internalize all of the EGF-R. Alternatively, the receptors could be competing for some as yet unidentified “internalization component” involved in the rapid internalization of occupied receptors. To decide between these two possibilities, it was necessary to establish whether high levels of occupied EGF-R block the internalization of other, nonrelated receptors. If high levels of occupied EGF-R interfered with EGF-R only, then some specific aspect of that receptor pathway must be involved. I decided to use the transferrin receptor (TF-R) as an alternate receptor system since it has been well characterized and enters coated pits along with EGF-R (Hanover et al., 1984; Hopkins et al., 1985).

I first characterized the dynamics of TF-R in A431 cells to establish a baseline by which to compare any effect of occupied EGF-R. Cells were incubated with concentrations of TF ranging from \( 5 \times 10^{-10} \) to \( 1 \times 10^{-8} \) M for 15 min in the presence of \( ^{125}\text{I}-\text{TF} \) to determine fluid uptake rates. Simultaneously, I incubated another group of cells with the same concentration range of \( ^{125}\text{I}-\text{EGF} \) for the same length of time to determine average receptor occupancies. The results of this experiment are shown in Fig. 7. Surprisingly, there was a direct relationship between receptor occupancy and the rate of fluid uptake at all levels of occupied EGF-R. There was no evidence of saturation of either net endocytosis or the ability of an occupied EGF receptor to stimulate a cellular response. I therefore conclude that the low specific internalization rate of occupied EGF-R at high occupancies is not a result of a functionally compromised cell. Instead, the occupied receptors must somehow be interfering with each other with respect to endocytosis.

![Figure 7](image-url)

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I next determined whether the presence of varying levels
of occupied EGF-R would affect the ability of Tf-R to be internalized. The results of this experiment are presented in Table I and show that the addition of high concentrations of EGF had no significant effect on the rate of Tf-R internalization. There was a small decrease (14%) in $k_o$ at the highest concentration of EGF, but this seems to be due to the correspondingly small increase (14%) in the average number of surface Tf-R induced by the EGF treatment. I have previously demonstrated that EGF stimulates the translocation of intracellular Tf-R to the cell surface, but the internalization of these newly inserted receptors is temporarily inhibited (Wiley and Kaplan, 1984). Thus high levels of occupied EGF-R in A431 cells do not interfere with the internalization of all surface receptors that use the coated pit pathway. Instead, it seems likely that the EGF-R are specifically interfering with each other.

**The EGF-R in A431 Cells Cannot Effectively Undergo Endocytic Downregulation**

One of the most well-established aspects of the EGF-R pathway is "endocytic downregulation", which is the rapid loss of the majority of surface EGF-R due to treatment with EGF (Carpenter and Cohen, 1976b; Aharonov et al., 1978; Wiley and Cunningham, 1982a; Anderson and Kaplan, 1983; Wiley, 1985). Although the regulatory significance of this process is still not certain, its mechanistic basis has been established to be due to an enhanced rate of internalization of occupied vs. unoccupied EGF-R (Wiley and Cunningham, 1981; Knauer et al., 1984). However, the results described above indicate that A431 cells are unable to efficiently internalize occupied EGF-R and thus should be unable to undergo rapid endocytic downregulation. I decided to determine if this was indeed the case. Since it is very difficult to accurately measure absolute receptor number in this cell type due to their occupancy-dependent binding properties, I decided to examine relative receptor number by using kinetics. Cells were treated with $8.3 \times 10^{-9}$ M EGF for 5, 30, and 120 min. The EGF was removed for 15 min after which they were cooled to 0°C and evaluated for their ability to bind $^{125}$I-EGF. As shown in Fig. 9, pretreatment with a high concentration of EGF had no significant effect on their subsequent ability to bind the ligand. The equilibrium binding constants determined from these data by curve-fitting were $8.3 \times 10^4$ M$^{-1}$ s$^{-1}$ for $k_o$ and $4.7 \times 10^{-4}$ s$^{-1}$ for $k_d$. These values yield an apparent equilibrium dissociation constant ($K_D = k_d/k_o$) of $5.6 \times 10^{-9}$ M, which is almost identical to the reported value of the low affinity receptors in this cell type (Kawamoto et al., 1983; Rees et al., 1984; Chatelier et al., 1986). As a comparison, when I treated normal human fibroblasts in parallel with $1.6 \times 10^{-9}$ M EGF for the same 5-, 30-, and 120-min periods followed by the 15-min recovery period, they lost 5, 58, and 87% of their initial complement of receptors (data not shown). I conclude from this experiment that as predicted, A431 cells are unable to effectively undergo rapid endocytic downregulation.

**Discussion**

The data presented here show that there are at least two major differences between the EGF receptor pathway in A431 cells and that of other cell types. The most unusual aspect is the apparent diffusion-limited binding of EGF at low receptor occupancies. Diffusion-limited binding is a predicted aspect of receptor systems that was not appreciated until the theoretical work of Berg and Purcell (1977) demonstrated how the presence of a membrane can significantly increase the capture efficiency of a receptor. Most experimental attempts to show diffusion-limited binding behavior have been negative, probably because it requires both a high intrinsic capture efficiency of a receptor (high forward rate constant) as well as sufficiently high surface receptor density (DeLisi and Wiegel, 1981; Wank et al., 1983). Recently, Erickson and his colleagues (1987) have provided the first experimental demonstration of the effect of receptor density on ligand binding. They confirm the prediction that the binding of a ligand to a cell will be diffusion controlled when the following relationship is true (DeLisi and Metzger, 1976).

$$Nk_i > 4\pi D_{ro},$$  (1)
where \( k_1 \) is the intrinsic forward rate constant for the receptor site–ligand binding, \( N \) is the number of receptors per cell, \( D \) is the diffusion coefficient of the ligand, and \( r_0 \) is the sum of the radii of the cell and ligand (assuming a spherical cell and a uniform distribution of receptors). The intrinsic forward rate constant of the human EGF receptor can be calculated to be \( \approx 5 \times 10^{-15} \text{ cm}^2 \text{s}^{-1} \) using the value of its association rate constant at low surface densities (Hock and Hollenberg, 1980; Knauer et al., 1984). If we estimate the diffusion coefficient of EGF as \( 15 \times 10^{-7} \text{ cm}^2 \text{s}^{-1} \) and the radius of an A431 cell as 5 \( \mu \text{m} \), then the right side of Eq. 1 becomes \( 9.4 \times 10^{-9} \text{ cm}^2 \text{cell}^{-1} \text{s}^{-1} \). Thus binding to cell surface EGF receptors can be predicted to become diffusion-limited when receptor number exceeds \( 1.9 \times 10^9 \) receptors per cell. However, Eq. 1 assumes a uniform receptor distribution. If EGF receptors are preclustered or heterogeneously distributed on the cell surface (which has been reported to be the case; Carpentier et al., 1982), then this would have the effect of raising the local receptor concentration and thus decrease the absolute number of receptors needed to observe diffusion-limited binding. For example, if EGF-R were preaggregated in groups of four, diffusion-limited binding would be observed with fewer than a million receptors (Van Opheusden et al., 1984). Therefore, the number of EGF receptors on A431 cells is sufficient to give rise to diffusion-limited binding unless a large percentage are occupied.

Because of the large number of receptors on A431 cells and the strong effect of receptor clustering on diffusion-limited binding, it is very difficult to determine the true surface distribution of receptors in that cell type. Current techniques for determining cell surface receptor distributions use the binding of high molecular weight, electron-opaque ligands at low temperatures. This technique is based on the assumption that the amount of ligand bound to a given surface region will be directly proportional to the local density of receptors. However, if the density is above that required for diffusion-limited binding, then the initial amount of bound ligand in any given region will be proportional to ligand diffusion rates and not to receptor density (Berg and Purcell, 1977; DeLisi and Wiegel, 1981). This problem would be exacerbated by using very large (and hence slowly diffusing), EGF derivatives. Thus the initially uniform binding of EGF derivatives to the surface of A431 cells observed at 4°C (Haigler et al., 1979a; McKanna et al., 1979, Hopkins et al., 1985) does not necessarily reflect the initial receptor distribution, but could instead demonstrate the diffusion-limited nature of the initial binding and the slow dissociation of EGF from its receptor at low temperature. The observation that warming the cells induces an apparent clustering of the bound ligand (Haigler et al., 1979a; McKanna et al., 1979; Hopkins et al., 1985) may not reflect a receptor redistribution induced by EGF binding, but instead be an illusion caused by lateral diffusion of ligand–receptor complexes and rebinding of dissociated ligand (the lack of apparent dissociation from A431 cells observed in Fig. 3 is due to rapid rebinding of dissociated ligand to previously unoccupied receptors). Therefore, the “clustered” state of the EGF-R “induced” by warming the cells could actually be the true steady state distribution of the receptors before ligand addition. The diffusion-limited nature of the initial binding could simply prevent one from observing this unless the ligand was given an opportunity to redistribute among the surface receptors by the process of dissociation and rebinding at the higher temperatures. It is perhaps significant that the only reported study in which a low molecular weight derivative of EGF (125I-EGF) was used to determine receptor distribution on cells with low receptor densities reported a preclustered state for the receptor (Carpentier et al., 1982).

A second major difference between the behavior of the EGF-R in A431 cells and fibroblasts is the apparent saturation of EGF-R internalization at high occupancies. This cannot be due to the presence of different affinity classes of receptors for two reasons. First, there is no significant difference in either the apparent forward or reverse rate constants of the EGF receptor at either \( 1.7 \times 10^{-11} \text{ m} \) or \( 1.7 \times 10^{-9} \text{ M} \) EGF: a range that spans both the reported equilibrium constants of high and low affinity receptors (Krupp et al., 1982; Kawamoto et al., 1983; Gamou et al., 1984; Rees et al., 1984; Chatelier et al., 1986) and high and low internalization rates (Fig. 4). Second, the internalization of any putative high affinity receptor can be readily blocked by adding unlabeled EGF (Fig. 6), indicating that rapid internalization is not a stable characteristic of any receptor subpopulation. The inhibition of EGF-R internalization is also not due to any general inhibition of endocytosis since fluid phase endocytosis increases continuously as a function of receptor occupancy (Fig. 7). Finally, the inhibition of EGF-R internalization cannot be due to an overloading of the endocytic system by occupied EGF-R since the rapid internalization of transferrin receptors is unaffected by high concentrations of EGF (Table 1). Together, my data suggest that occupying high numbers of EGF-R specifically interferes with EGF-induced EGF-R internalization.

There are two reasonable explanations as to how high numbers of occupied receptors could interfere with each other. The simpler is that self-aggregation of receptors inhibits their internalization by coated pits. Schlessinger and co-workers have accumulated evidence using A431 cells that receptor aggregation/clustering is a consequence of EGF binding (Zidovetzki et al., 1981; Hillman and Schlessinger, 1982). They interpreted those data as suggesting that receptor clustering was necessary for internalization. Although they measured internalization rates at low EGF concentrations, receptor aggregation was determined using ligand concentrations that inhibit internalization (Zidovetzki et al., 1981). Hopkins et al. (1985) have also presented data that suggests high levels of occupied EGF-R in A431 cells leads to a clumping of receptors outside of coated pits. Thus it is possible that the widely cited evidence for the involvement of receptor clustering in EGF-R internalization could instead explain the inability of high numbers of occupied EGF-R to be efficiently internalized. However, a difficulty with this hypothesis is that it does not explain why the specific internalization rates of EGF-R invariably reach a constant lower value at high occupancy levels instead of approaching zero. Nevertheless, it is possible that some aspect of receptor aggregation could be involved in inhibiting EGF-R internalization.

A second explanation is that A431 cells lack sufficient numbers of a component that mediates the rapid, occupancy-
induced internalization of EGF-R. In this scenario, A431 cells would synthesize an abnormally large number of receptors, but only a normal complement of other proteins/components involved in receptor internalization or regulation. In this respect A431 cells would be similar to Xenopus oocytes which have enormous numbers of surface receptors for the yolk precursor protein, vitellagenin (<1 × 10^{11} per cell), but do not have corresponding levels of a hormonally regulated component that mediates rapid internalization (Opresko and Wiley, 1987b). As was the case with oocytes, treatment of A431 cells with serum preferentially stimulates receptor internalization at low occupancies (unpublished observations). If there is a currently unidentified (membrane?) component that specifically mediates the rapid, occupancy-induced internalization of the EGF-R, then it would probably act by binding to occupied (phosphorylated?) EGF-R and facilitating its entry into the coated pit pathway. At high receptor occupancies, this component could associate with only a fraction of the occupied receptors, forcing most to be internalized by the constitutive route taken by empty receptors. The slow internalization of the majority of receptors would in effect obscure the rapid internalization of the minority of receptors able to bind to the "internalization component." Supporting this hypothesis is the demonstration that high concentrations of EGF do not effectively change the number of receptors observed in the absence of ligand (Fig. 9), indicating that most receptors are being internalized by the constitutive pathway involved in normal membrane turnover. However, it is difficult to predict whether any such internalization component would be specific for the EGF-R or might function as a mediator for a class of receptor types. Further studies on the ability of EGF-R to compete for internalization with a variety of different receptor types in A431 cells would probably prove most interesting.

This study was initiated to determine the degree to which receptor studies on A431 cells can be extrapolated to other cell types. I felt it was essential to have this type of information especially in the light of some recent studies on A431 cells that have found significant differences in behavior between the class 1 EGF-R and the class 2 Tf-R (Hopkins et al., 1985). The initial random distribution of the EGF-R and the preclustered nature of the Tf-R were interpreted as reflecting intrinsic differences between those receptors undergoing endocytic downregulation and those being constitutively internalized (Hopkins et al., 1985). However, my studies indicate that the observed differences between the two receptor types could be a reflection of the relatively normal behavior of the Tf-R and the very unusual properties of the EGF-R in A431 cells. Pronounced differences in the distribution of EGF-R and the low density lipoprotein receptor have not been observed in human fibroblasts (Carpentier et al., 1982). However, as is the case with EGF-R, the behavior of low density lipoprotein receptors is significantly different between fibroblasts and A431 cells (Anderson et al., 1981). Thus it is not at all certain what constitutes the normal distribution or behavior of different classes of receptors, especially since the relationship between receptor density/distribution in ligand binding is complex.

Although A431 cells are an atypical cell for investigating the surface dynamics of the EGF-R, they could be an extremely useful system for investigating receptor regulation. For example, I was very surprised to find that the induction of fluid-phase endocytosis was directly proportional to receptor occupancy at all levels (Fig. 7). This direct correlation between receptor occupancies and responses in A431 cells has previously been observed with respect to induced calcium influx (Sawyer and Cohen, 1981). Paradoxically, cells with a normal complement of receptors show a strong attenuation in EGF-R-mediated responses at high occupancies, presumably through a feedback loop (Wiley and Cunningham, 1982b; Wiley and Kaplan, 1984). Thus, in addition to lacking a sufficient complement of components necessary for the rapid internalization of the EGF-R, A431 cells may also lack an adequate number of regulatory molecules for regulating EGF-R mediated cellular responses. This could explain the known cytotoxic effects of high concentrations of EGF (Gill and Lazar, 1981; Barnes, 1982; Gamou et al., 1984). Supporting this hypothesis is the observation that although the UCVA-1 cell line has high numbers of EGF-R, their response to EGF is strongly attenuated relative to A431 cells and EGF is not toxic (Gamou et al., 1984). Thus the knowledge that A431 cells are highly unusual with respect to their binding and internalization of EGF does not invalidate their use as an important experimental system. However, an understanding of their behavior in the context of the total range of possibilities is necessary to fully exploit the experimental advantages that they have to offer.

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