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 4×10^3 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)[1] 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

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1. *Abbreviations used in this paper:* EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; k_e, endocytic rate constant; PVP, polyvinylpyrrolidone; Tf, diferric transferrin; Tf-R, transferrin receptor.

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, Carpentier 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 diferric 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 ^{125}I 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 ^{125}I-labeled EGF was generally between 760 and 1,700 cpm/fmol whereas that of the ^{125}I-labeled Tf was between 200 and 400 cpm/fmol.

The A431 cell line was obtained frm 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 × 10⁶ 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 glutaldehyde as described (Moses et al., 1980). The fluid-phase marker ^{125}I-polyvinylpyrrolidone (PVP) was prepared by the UV-catalyzed oxidative reaction as previously described (Wiley and McKinley, 1987). The specific activities were generally around 1 × 10⁹ 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 ^{125}I-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 ^{125}I-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 (k_e) 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 k_e were created for both SuperCalc 3a (Apple IIe 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 ^{125}I-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 × 10⁴, 1.6 × 10⁵, and 6.6 × 10⁵ 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 × 10⁴ M⁻¹ s⁻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).

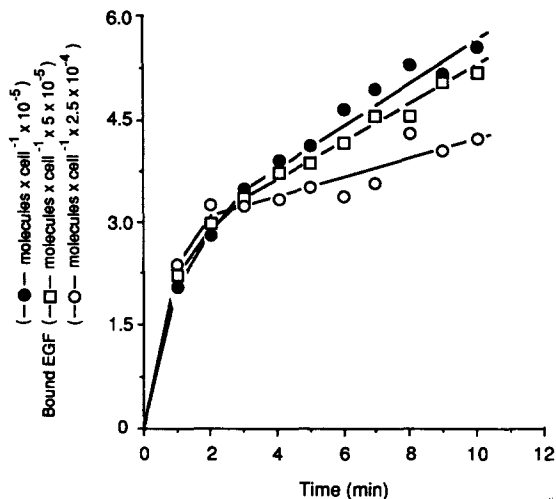The kinetics of EGF binding to the surface of A431 cells

Figure 1. Kinetics of surface binding of ^{125}I-EGF in A431 cells. Confluent monolayers of cells were incubated with ^{125}I-EGF at concentrations of 1.7 × 10^{-10} M (o), 8.3 × 10^{-10} M (□), or 4.1 × 10^{-9} M (●) 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.

are quite different from the "classic" binding seen in human fibroblasts. Presented in Fig. 2 is the first 10 min of binding of EGF to the surface of normal human fibroblasts (with ~1 × 10^5 receptors/cell) using a 16-fold concentration range. As in Fig. 1, the y-axis of the figure has been adjusted to compensate for the different ligand concentrations. The time necessary to reach half maximal surface binding is greater at the lower ligand concentrations (1.2, 1, and 0.9 min for 1.3 × 10^{-10}, 5.3 × 10^{-10}, and 2.1 × 10^{-9} M EGF, respectively). The number of receptors lost by a combination of occupancy and ligand-induced internalization for the three
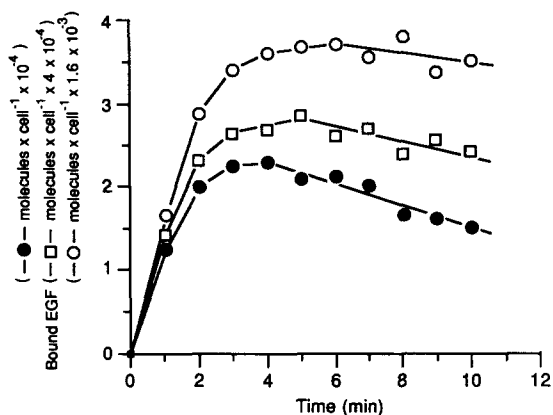

Figure 2. Kinetics of surface binding of ^{125}I-EGF in normal human fibroblasts. Confluent monolayers of cells were incubated with ^{125}I-EGF at concentrations of 1.3 × 10^{-10} M (o), 5.3 × 10^{-10} M (□), or 2.1 × 10^{-9} M (●) and at the indicated times the amount of ligand associated with the surface of the cells was determined by acid stripping as described in Materials and Methods. As was done in Fig. 1, the scales for the different curves were adjusted to factor out the relative ligand concentration as indicated along the y-axis.

different ligand concentrations was 6.6 × 10^3, 2.0 × 10^4, and 5.5 × 10^4 receptors per cell, respectively. The relative extent of surface binding is also reduced at the higher ligand concentrations. These features are typical for most ligand-receptor interactions. However, it is remarkable that a reduction in the number of empty surface EGF-R in A431 cells by over 600,000 per cell was accompanied by a relative increase in surface binding (Fig. 1). This observation together with the linear binding kinetics, the linear dependence of binding on ligand concentration, and the very low "apparent" second-order rate constant suggests that binding of EGF to A431 cells is a diffusion-limited process (Berg and Purcell, 1977; DeLisi and Wiegel, 1981; Wank et al., 1983).

In typical ligand-receptor reactions, the net rate of binding is determined by ligand-receptor concentrations as well as by steric and orientation factors that determine how rapidly a ligand molecule in the proximity of receptor will fit into the binding site. However, if the density of receptors and their capture efficiency are sufficiently high, the potential rate of ligand binding in a given area of a membrane will exceed the rate at which the ligand can diffuse (Berg and Purcell, 1977). The rate of binding then becomes a diffusion-limited process in which receptors effectively compete with each other for ligand capture.

Diffusion-limited binding is characterized by a rate of ligand dissociation which is very dependent on the number of empty receptors. This is because dissociation is functionally measured as the ability of a released ligand to diffuse away from the membrane without being recaptured by a neighboring empty receptor (DeLisi and Wiegel, 1981; Wank et al., 1983). If binding is diffusion-limited, then the probability that a neighboring receptor will capture the ligand is much greater than its probability of escaping by diffusion. To examine the occupancy-dependent dissociation of EGF, I used membranes isolated from A431 cells. Membranes were incubated at 37°C with 1.7 × 10^{-9} M ^{125}I-EGF for 2 h and then diluted 10 times with buffer containing various concentrations of unlabeled EGF. If rapid rebinding of dissociated ^{125}I-EGF was occurring, then blocking empty receptors by adding unlabeled EGF should accelerate the loss of previously bound ligand. As shown in Fig. 3, the apparent rate of ^{125}I-EGF dissociation was indeed accelerated by the addition of increasing amounts of unlabeled ligand. However, above a concentration of 2 × 10^{-7} M unlabeled EGF, the rate constant of dissociation reached a limiting value of ~1.8 × 10^{-3} s^{-1} which is half of that observed in human fibroblasts (3.6 × 10^{-3} s^{-1}; results not shown). In the absence of unlabeled EGF, <10% of the ^{125}I-EGF had dissociated by 1 h (k_d <3 × 10^{-5} s^{-1}). I also tested the effect of anti-EGF antiserum on ^{125}I-EGF dissociation since this should also accelerate ^{125}I-EGF dissociation by acting as an alternate sink for dissociated ligand and thus competing with the empty receptors. As shown in Fig. 3, anti-EGF antiserum also accelerated the dissociation of previously bound ligand. Although very high concentrations of antiserum were required, increasing amounts did increase the rate of ligand dissociation up to a limiting value of 1.8 × 10^{-3} s^{-1} (data not shown). The same concentrations of antiserum had no detectable effect on the dissociation of ^{125}I-EGF from human fibroblasts, indicating that the antibodies are not simply disrupting the ligand-receptor complex (results not shown). These data indicate that at low levels of receptor occupancy,
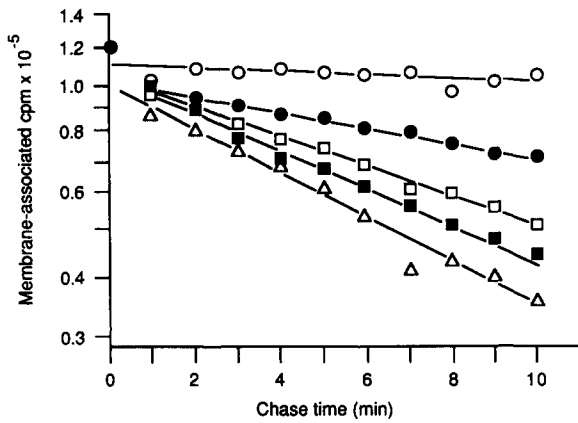
Figure 3. The addition of either unlabeled EGF or anti-EGF antibodies to A431 cell membranes accelerates the dissociation of previously bound ^{125}I-EGF. A431 cell membranes were prepared and incubated to apparent equilibrium at 37°C with 1.7 × 10^{-9} M ^{125}I-EGF as described in Materials and Methods. They were then diluted 10-fold with buffer containing either none (O), 1.7 × 10^{-9} M (□), 8.3 × 10^{-9} M (■), or 1 × 10^{-6} M (△) unlabeled EGF. Alternately, the dilution buffer contained a 10:1 dilution of anti-EGF antiserum (●). At the indicated time points, the samples were filtered and rinsed four times at 0°C. The addition of equivalent amounts of unrelated antisera or ligands had no effect on the rate of ^{125}I-EGF dissociation.

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 ^{125}I-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 phe-
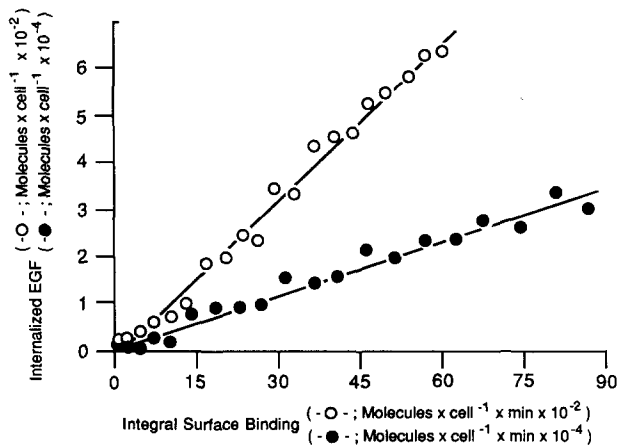
nomenon, I incubated cells with either 1.7 × 10^{-11} or 1.7 × 10^{-9} M ^{125}I-EGF and then determined the relative amount of internalization by acid stripping. The data were then used to generate the internalization plot shown in Fig. 4. In an internalization plot, the slope of the curve at any point 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 × 10^{-9} M ^{125}I-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}I-EGF concentrations ranging from 8.3 × 10^{-12} to 1.6 × 10^{-8} 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 ∼5 × 10^5 receptors

Figure 4. Internalization plots of the uptake of ^{125}I-EGF by A431 cells. The kinetics of the first 10 min of surface binding and internalization of either 1.7 × 10^{-11} M (O) or 1.7 × 10^{-9} M (●) ^{125}I-EGF was determined at 30-s intervals by acid stripping and then converted to an internalization plot as described (Opresko and Wiley, 1987a,b). The slope of the plot at any point is equal to the average specific internalization rate of the occupied receptors at that time. The values obtained were 0.14 and 0.05 min^{-1} for 1.7 × 10^{-11} and 1.7 × 10^{-9} M ^{125}I-EGF, respectively.
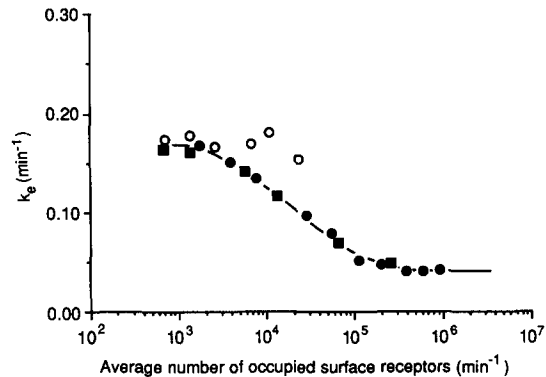

Figure 5. The specific internalization rate of the EGF-R saturates at high receptor occupancies. The kinetics of ^{125}I-EGF internalization in A431 cells was measured for up to 15 min using ^{125}I-EGF concentrations from 8.3 × 10^{-12} to 1.7 × 10^{-8} M. The relative distribution of label between the surface and the inside of the cells was determined and converted to internalization plots as described (Opresko and Wiley, 1987a, b). The values obtained from these plots (the specific internalization rate of the EGF-R) are shown as a function of the average number of receptors occupied during the entire period of uptake. The solid symbols are results obtained from two separate experiments whereas the open circles are results obtained from performing the identical experiment on normal human fibroblasts.

min^{-1}. In contrast, fibroblasts displayed a specific internalization rate which was independent of receptor occupancy (Fig. 5).

The only other receptor system reported to date that also demonstrates a "saturable" internalization is the vitellogenin receptor in *Xenopus* oocytes (Opresko and Wiley, 1987a, 1987b). That cell type also displays enormous receptor densities and shows the same occupancy-dependent inhibition of k_e as shown in Fig. 5. One distinguishing feature of oocytes is that the high internalization rates seen at low receptor occupancies can be increased by hormonal treatment whereas the low internalization rates at higher occupancies are insensitive to hormones (Opresko and Wiley, 1987b). To determine if such a situation was also true with A431 cells, I repeated the above dose–response experiments, but included 5% FCS in the preincubation medium as a general inducer of hormonal responses. This resulted in a small, but reproducible 25% increase in k_e at low receptor occupancies (from 0.16 to 0.20 min^{-1}), but had no effect on k_e at higher occupancies. In this respect, the endocytic system system in A431 cells is similar to that observed for *Xenopus* oocytes.

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 one 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 ^{125}I-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 ^{125}I-EGF from the cells since all of the ^{125}I-EGF lost from the cell surface appeared inside the cells. In
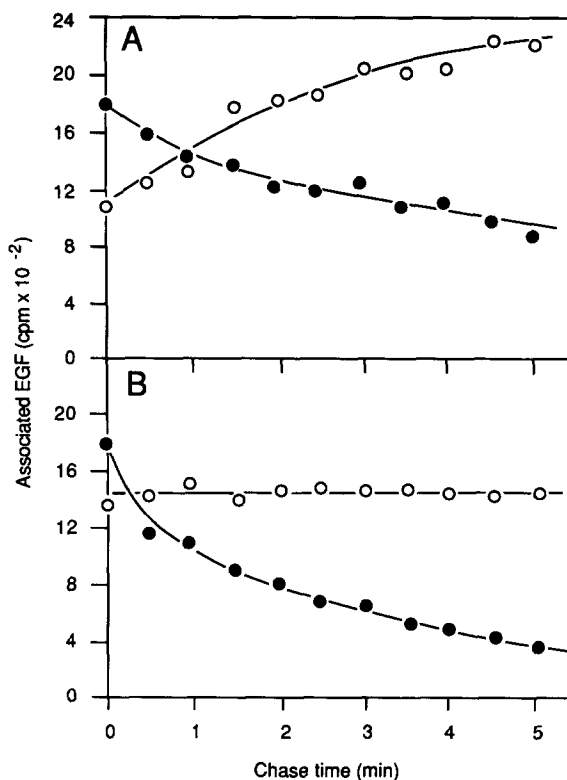

Figure 6. The addition of high concentrations of EGF to A431 cells inhibits the internalization of previously occupied receptors and accelerates the loss of ^{125}I-EGF from the cell surface. Confluent monolayers of cells were incubated for 5 min with 1.7 × 10^{-11} M ^{125}I-EGF at 37°C. The labeled ligand was removed and replaced with medium containing (*A*) an equal amount of unlabeled EGF or (*B*) a much higher concentration (8.3 × 10^{-7} M). The relative amount of label associated with either the surface (●) or inside the cells (○) was determined by acid stripping. There was no degradation of ^{125}I-EGF observed during the experiment as determined by native gel electrophoresis (Wiley and Cunningham, 1982).

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 ^{125}I-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 ^{125}I-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-
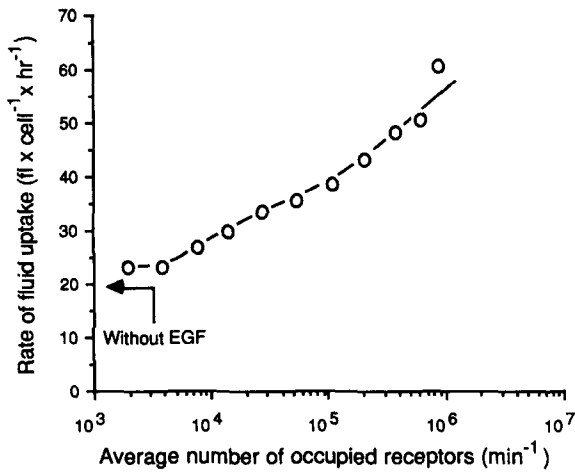
Figure 7. EGF-stimulated fluid phase endocytosis is proportional to receptor occupancy at all concentrations of EGF. Confluent monolayers of A431 cells were incubated with ^{125}I-PVP and unlabeled EGF concentrations ranging from 1.7 × 10^{-11} to 1.7 × 10^{-8} M. The amount of ^{125}I-PVP internalized 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}I-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.

Figure 8. The specific internalization rate of the Tf-R in A431 cells is independent of receptor occupancy. Confluent monolayers of cells were incubated with 5 × 10^{-10} M (◇), 1 × 10^{-9} M (○), 5 × 10^{-9} M (□), or 1 × 10^{-8} M (■) ^{125}I-Tf for 10 min at 37°C. At 1-min intervals the relative amount of ligand associated with the surface and the inside of the cells was determined by acid stripping as described in Materials and Methods. The data were then converted into an internalization plot as described (Opresko and Wiley, 1987a,b). Note that despite the wide difference in ligand concentrations, all of the data points fall on the same straight line, yielding a value of 0.55 min^{-1} for k_e.

curs as a result of EGF-stimulated membrane translocation to the cell surface (Wiley and Cunningham, 1982b; Wiley and Kaplan, 1984). Since fluid phase endocytosis will reveal both the net ability of the cells to internalize membrane and also the ability of the cells to respond to occupied EGF receptors, it seemed an ideal assay to test for any "paralysis" that may occur as a result of high receptor occupancies. Cells were treated with concentrations of EGF ranging from 1.6 × 10^{-12} to 1.6 × 10^{-8} M for 15 min in the presence of ^{125}I-PVP to determine fluid uptake rates. Simultaneously, I incubated another group of cells with the same concentration range of ^{125}I-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.

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. Alter-
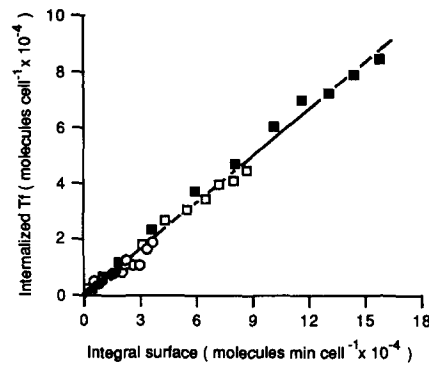
nately, the receptors could be competing for some as yet unidentified "internalization component" involved in the rapid internalization of occupied receptors. To decide between these two possibilities, it was necessary to establish whether high levels of occupied EGF-R block the internalization of other, 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 ^{125}I-Tf ranging from 5 × 10^{-10} to 1 × 10^{-8} M and the relative distribution of ligand between the surface and inside of the cells was measured at 1-min intervals. The data were then converted into the internalization plots shown in Fig. 8. There are three important aspects of this graph. The first is that all of the curves are superimposable, indicating that the specific internalization rate of Tf-R in A431 cells is independent of receptor occupancy (compare this with Fig. 4). The second is that the maximum number of occupied receptors falls within the range of "normal" cells (maximum average number of occupied receptors about 2 × 10^4 min^{-1}). The third is that Tf-R is internalized very rapidly in these cells. In a series of five experiments, the specific internalization rate for Tf-R ranged between 0.48 and 0.96 min^{-1} which is about three to six times faster than the maximum value of EGF-R. It is also several times faster than the rate of Tf-R internalization in fibroblasts (Wiley and Kaplan, 1984). This indicates that A431 cells have at least one receptor system that is very efficiently internalized and is present at approximately normal levels.

I next determined whether the presence of varying levels

Table I. Effect of EGF on Transferrin Internalization

EGF concentration	Average surface ^{125}I-Tf	k_e of Tf receptor
M	*fmol/10^6 cells*	*min^-1*
0	2.8	0.97
1.6×10^{-10}	2.8	0.95
1.6×10^{-9}	2.9	0.91
1.6×10^{-8}	3.2	0.83

Confluent cultures of A431 cells were incubated with the indicated concentration of EGF and ^{125}I-Tf at 10^{-9} M for up to 10 min at 37°C. At 1-min intervals the cells were rinsed and the amount of ^{125}I-Tf associated with either the surface or inside of the cells determined by acid stripping and corrected for stripping efficiency and nonspecific binding. The specific internalization rate of the Tf receptor and the average surface binding of ^{125}I-Tf was then determined by using internalization plots. The correlation coefficients of all internalization plots were >0.99.
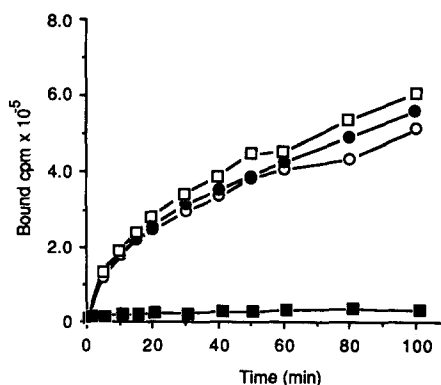

Figure 9. EGF receptors in A431 cells do not effectively undergo endocytic downregulation. Confluent monolayers of cells were treated at 37°C with 8.3×10^{-9} M EGF for 5 min (□), 30 min (●), or 120 min (o). The cells were switched to prewarmed medium lacking EGF for 15 min to allow surface-associated EGF to be lost by internalization/dissociation. The cells were then rapidly rinsed with ice-cold saline and incubated at 0°C with 1.7×10^{-9} M ^{125}I-EGF for the indicated periods of time at which point the amount of radiolabeled ligand associated with the cells was determined. Also included in this figure is the nonspecific binding obtained in the presence of 8.3×10^{-7} M unlabeled EGF (■).

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_e 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 8.3×10^{-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 8.3×10^4 M^{-1} s^{-1} for k_a and 4.7×10^{-4} s^{-1} for k_d. These values yield

an apparent equilibrium dissociation constant ($K_D = k_d/k_a$) of 5.6×10^{-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 1.6×10^{-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 collegues (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).

$$Nk_1 > 4\pi Dr_0, \tag{1}$$

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 $\sim 5 \times 10^{-15}$ cm^2 s^{-1} using the value of its association rate constant at low surface densities (Hock and Hollenberg, 1980; Knauer et al., 1984).[2] If we estimate the diffusion coefficient of EGF as 15×10^{-7} cm^2 s^{-1} and the radius of an A431 cell as 5 μm, then the right side of Eq. 1 becomes 9.4×10^{-9} cm^2 cell^{-1} s^{-1}. Thus binding to cell surface EGF receptors can be predicted to become diffusion-limited when receptor number exceeds $\sim 1.9 \times 10^6$ 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 (^{125}I-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 1.7×10^{-11} m or 1.7×10^{-9} 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 inhibiton 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 I). 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-

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2. This is the average k_1 per receptor molecule calculated as $k_a(\text{M}^{-1}\text{s}^{-1})$ $\times$ 1,000/6.02 $\times$ 10^{23}.

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, vitellogenin ($\leq 1 \times 10^{11}$ 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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