Kinetics of Binding, Endocytosis, and Recycling of EGF Receptor Mutants

S. Felder,* J. LaVin,* A. Ullrich,‡ and J. Schlessinger§

*Department of Molecular Biology, Rhone-Poulenc Rorer, Inc., King of Prussia, Pennsylvania 19406; ‡Max-Planck-Institut fur Biochemie, 8033 Martinsreid bei Munchen, Germany; and §Department of Pharmacology, New York University Medical Center, New York 10016

Abstract. This report describes analysis of factors which regulate the binding of EGF to EGF receptor, receptor internalization, and receptor recycling. Three different methods were used to inhibit high-affinity EGF binding as measured at equilibrium: treatment of cells with an active phorbol ester (PMA), binding of a mAb directed against the EGF receptor (mAb108), and truncation of most of the cytoplasmic domain of the receptor. These treatments reduced the rate at which low concentrations of EGF bound to cells, but did not affect the rate of EGF dissociation. We conclude that high-affinity EGF binding on living cells results from a difference in the apparent on rate of EGF binding. We then used these conditions and cell lines to test for the rate of EGF internalization at different concentrations of EGF. We demonstrate that internalization of the EGF receptor is stimulated roughly 50-fold at saturating concentrations of EGF, but is stimulated an additional two- to threefold at low concentrations (<1 nM). Four treatments reduce the rate of internalization of low concentrations of EGF to the rate seen at saturating EGF concentrations. Phorbol ester treatment and mAb108 binding to "wild type" receptor reduce this rate (and reduce high-affinity binding). Point mutation at Lys721 (kinase negative EGF receptor) and point mutation at Thr654 (removing a major site of protein kinase C phosphorylation) reduce the internalization rate, without affecting high-affinity binding. We suggest that while EGF stimulates endocytosis for all receptors, high-affinity receptors bind and are internalized more quickly than low-affinity receptors. Tyrosine kinase activity and the Thr654 region appear necessary for this response.

When EGF binds to its receptor (EGFR)[1] on the surface of target cells, several early and late responses occur (Carpenter and Cohen, 1979) all of which are dependent upon activation of the tyrosine-specific kinase intrinsic to the receptor (Honegger et al., 1987; Chen et al., 1987; Moolenaar et al., 1988; reviewed in Ullrich and Schlessinger, 1990). In addition to the physiological responses, the receptor and bound EGF are rapidly internalized and degraded. Several articles have recently appeared analyzing the kinetics of binding and internalization of EGF for normal and mutant EGFR (Bellot et al., 1990; Waters et al., 1990; Berkers, et al., 1991). Some of these have presented disparate views of these events. Our previous results showed that for normal EGFR the initial rate of EGF binding is higher at lowest (<1 nM) EGF concentrations. Further, under conditions favoring either high-affinity or low-affinity binding the rates of dissociation of EGF were the same (Bellot et al., 1990). This suggests that high-affinity binding results from an elevated on-rate for high-affinity receptors as compared to that for low-affinity receptors, that is, that high-affinity binding is controlled by the rate of association rather than the rate of dissociation. Others have reported that the rate of EGF dissociation can vary under different conditions including length of preincubation (Waters et al., 1990). Recently our view was supported by data demonstrating that EGF associates more quickly to cells in the presence of a mAb 2E9, which specifically reduces low-affinity EGF binding (Berkers et al., 1991).

Following binding, receptor and bound EGF undergo rapid internalization followed by rapid degradation. We have previously shown that inactivation of the intrinsic kinase by point mutation has little effect on the rate of internalization of EGF and receptor, but reduces receptor down regulation because the kinase inactive receptor is recycled (Honegger et al., 1987; Felder et al., 1990). In contradiction to this conclusion, another group has suggested that kinase inactive receptor is not internalized (Glenney et al., 1988; Chen et al., 1989).

The present study testing the kinetics of binding and internalization for cells expressing normal and mutant human EGF receptors provides further evidence for recycling of ki-

1. *Abbreviations used in this paper*: ASGP, asialo glycoprotein; EGFR, epidermal growth factor-receptor; PMA, 4 beta-Phorbol 12-myristate 13-acetate.

nase negative EGF receptors. In addition, we find a connection between the elevation in rate of association at low EGF and an elevation in the apparent rate of internalization at low EGF which suggests that high-affinity EGF receptors are internalized more rapidly than are low-affinity receptors. Further, the elevated rate of internalization depends upon tyrosine kinase activity and on an intact Thr654 site.

Materials and Methods

Cell Lines

NIH-3T3 cells, clone 2.2, which express no endogenous EGFR were transfected with a plasmid bearing a cDNA for the human EGFR, either wild type (HER14s, expressing 300,000 receptors/cell), a point mutant kinase negative EGFR with lysine 721 replaced with alanine (K721A cells, 300,000 receptors/cell; Honegger et al., 1987), a point mutant with threonine 654 replaced with tyrosine (T654Y cells expressing 100,000 receptors/cell; Livneh et al., 1988), or a truncated receptor missing 533 amino acid residues from the carboxy terminus (CD533, expressing 1 million receptors/cell; Livneh et al., 1987).

Radiolabel Studies

For binding and internalization studies cells were plated on human fibronectin coated 35-mm dishes or 24-well plates (Costar, Cambridge, MA), and grown in DME with 10% calf serum. Either just below or at confluence, cells were incubated with mouse ^{125}I-EGF (iodinated with the Iodogen method to 1.5×10^{18} cpm/mol) in binding buffer: DME supplemented with 20 mM Hepes and 0.2% BSA. Where indicated cells were preincubated at 37°C for 30 min in binding buffer with 50 μM monensin (diluted from a 20 mM stock in methanol) or with 100nM PMA (diluted from a 100 μM stock in 10% DMSO), and kinetics incubations were done in the continued presence of monensin or PMA.

Kinetic studies of dissociation and association rates were performed as described previously (Bellot et al., 1990). Briefly, for dissociation experiments ^{125}I-EGF was prebound to cells at 4°C for 90 min. Time course experiments for all cell lines tested showed that binding reached plateau by 90 min even for the lowest EGF concentrations used (data not shown). Cells were then washed twice and left in binding buffer containing 50 nM unlabeled EGF at 4°C for various times. After incubation cells were washed twice with binding buffer, solubilized in 0.2 M KOH and counted. Parallel data taken with 1 μg/ml unlabeled EGF present during prebinding were subtracted from all data to correct for nonsaturable binding. Association experiments were done at 37°C. Cells were washed with warmed binding buffer and left to equilibrate to that temperature for 15 min. Buffer was then replaced with 1.0 ml of prewarmed binding buffer containing ^{125}I-EGF at desired concentrations. Association was followed for up to 10 min. After incubation cells were washed twice within 1 min with binding buffer at 4°C, solubilized and counted. Nonsaturable binding values determined in the presence of 1 μg/ml unlabeled EGF ranged from 5 to 20% of binding values and were subtracted from all values. The effect of internalization on the binding rates so analyzed was not considered for the following reasons. Internalization would not affect our measurement of binding, except by preventing dissociation. But during the first 10 min of binding, little dissociation would be expected: at the dissociation rate reported herein, 16% of surface bound EGF would dissociate in 10 min. In support of this prediction, measurements were checked in the presence of 20 μM phenylarsine oxide to prevent 90% of EGF internalization. Binding values differed by <10% from those without this drug.

Internalization studies were done without prebinding of radioligand in a similar fashion to the association experiments. Cells were equilibrated in binding buffer at 37°C for 10 min and buffer was replaced with prewarmed binding buffer containing ^{125}I-EGF to start the experiment. At various times cells were washed twice in 1 min with binding buffer at 4°C. Surface bound ^{125}I-EGF was assessed by collecting two washes of cells with PBS adjusted to pH 2 with HCl for a total of 3 min at 4°C. Internal ligand was assessed by dissolving cells and ligand remaining after pH 2 wash with 0.2 M KOH. Nonsaturable binding was determined as above and subtracted from all values.

Calculations and Fitting Procedures

Association data and internalization data were fit by a nonlinear least squares algorithm (Bevington, 1969) after solution of the fitting equations by numerical integration. Integrations were done at 1-s intervals by trapezoid rule, and results were not affected by changing the integration interval to 0.1 s. The time required for fitting each internalization data set was <2 min on a microVAX computer (Digital Equipment Corp., Marlboro, MA), including solution of a set of four related differential equations, at integrations of 1-s intervals out to 20 min, and allowing for five iterations of the least squares algorithm. Data from association experiments were fit as described previously (Bellot et al., 1990) to the differential equation presented in the legend to Table 1. Data from internalization experiments were fit to the following set of differential equations.

$$\frac{d(E{:}R)_s}{dt} = -k_e[E{:}R]_s + k_{ex}[E{:}R]_i + k_a[R]_s[E] - k_d[E{:}R]_s$$

$$\frac{d[E{:}R]_i}{dt} = k_e[E{:}R]_s - k_{ex}[E{:}R]_i$$

$$\frac{d[R]_s}{dt} = -k_a[R]_s[E] + k_d[E{:}R]_s$$

$$\frac{d[E]}{dt} = -k_a[R]_s[E] + k_d[E{:}R]_s$$

where:

k_e = apparent first order rate constant of endocytosis
k_{ex} = apparent first order rate constant of exocytosis (recycling)
$[R]_s$ = concentration of unoccupied receptor on the cell surface
$[E{:}R]_s$,$[E{:}R]_i$ = concentration of occupied receptor outside and inside
$[E]$ = concentration of EGF
k_a = apparent first order rate constant of association
k_d = apparent first order rate constant of dissocation

Values for the dissociation rate constants (k_d), and for the total receptor number, initial $[R]_s$, were estimated from association experiments for each cell line and were input as constants to the fitting procedure. The resultant estimated values for k_a, k_e, and k_{ex} were very little affected by changes in the value of k_d used. For example, for the data presented in Fig. 4 *b*, a fivefold increase in k_d from the value for which the chisquared was a minimum, yielded a 12 and a 17% decrease in estimated k_e and k_{ex}, respectively. A fivefold decrease in k_d, increased estimated k_e and k_{ex} by 2 and 6% only. Hence, the exact value of k_d at 37°C, even if it were not constant as the 4°C dissociation data suggested, would not have affected the estimation of these parameters.

Results

High-affinity Binding to Intact Cells Results from an Elevated Rate of Association

To test whether high-affinity binding of EGF to EGFR expressed on the surface of intact cells results from an increased rate of association or decreased rate of dissociation, we have measured the rate of dissociation (Fig. 1) and association (Fig. 2 and Table I) for three different cells, under different conditions. A431 cells are derived from an epidermoid carcinoma and overexpress the EGFR, having roughly 2 million receptors per cell. They have been widely used, and were used here for comparison and to maximize signal. The remaining cell lines were NIH-3T3 cells lacking endogenous EGFR (clone 2.2) transfected with the normal human EGFR, the HER14 cells (Honegger et al., 1987), or with mutant EGFRs. CD533 cells express a receptor truncated at residue 653, missing all except the first nine amino acids of the cytoplasmic, carboxy terminal domain (Livneh et al., 1986; Kashles et al., 1991). The truncated receptor binds EGF with low affinity only, with a K_d of 3–7 nM (Livneh et al., 1986). K721A express a point mutant tyrosine kinase inactive receptor for which lysine 721 was replaced with alanine (Honegger et al., 1987). T654Y express a point mutant for which threonine 654 was replaced with tyrosine (Livneh et al., 1988).

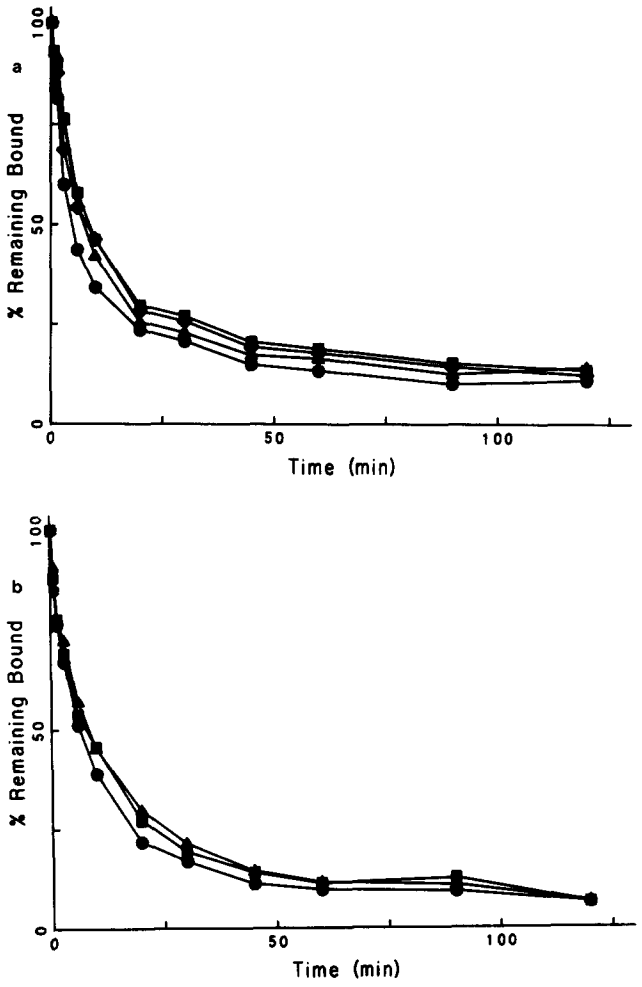

Figure 1. Dissociation of EGF. After prebinding of ^{125}I-EGF at 4°C, cells were washed and incubated with excess unlabeled EGF, and the amount of label remaining bound after different times of wash at 4°C are plotted, for A431 cells (*a*) and CD533 cells (*b*). Cells were used without pretreatments (■, ♦), pretreated with 100 nM PMA for 30 min at 37°C (▲), or prebound with 100 nM FAB' fragments of mAb108 (●). Cells were prebound with 10 nM ^{125}I-EGF for all data except for one data curve for A431 cells, for which 0.5 nM EGF was used (♦).

Fig. 1 displays EGF dissociation from A431 cells (Fig. 1 *a*) or from CD533 cells (Fig. 1 *b*) at 4°C in the presence of excess unlabeled EGF to prevent rebinding. For all cell lines and conditions tested the dissociation curves were little different. Similar experiments were performed without excess unlabeled EGF. The rates of dissociation were faster with EGF, by roughly fivefold. However, the conclusions were the same: for all conditions tested no significant differences in the off rates were seen. Specifically the dissociation of EGF for conditions which would be expected to reflect binding to high-affinity sites was not different from dissociation from cells or conditions which show no measurable high-affinity binding. The former conditions were dissociation of EGF from wild type receptor on either A431 or on HER14 (data not shown) cells at low receptor occupancy (~3% of receptors occupied). The latter conditions were deletion of the cytoplasmic domain, treatment with either phorbol ester (PMA) or a mAb directed against the extracellular domain of EGFR, mAb108 (Bellot et al., 1990), and dissociation of EGF from fully occupied receptors (which may reflect dissociation from the more numerous low-affinity sites). Further, the dissociation kinetics following 5 min or following 60 min of EGF prebinding were the same (data not shown). We note that the halftime of dissociation from low occupancy in the presence of mAb108 was increased ~1.5 to twofold. This difference was significant, but clearly could not account for inhibition of high affinity binding. At low EGF ~50% of bound EGF should result from binding to high affinity sites. If mAb108 were inhibiting these sites by affect on off-rate, then half of the bound EGF should have dissociated 10-fold more quickly. This clearly was not the case.

The rate of association of EGF with the cells at 37°C did differ with conditions. In agreement with data for the HER14 cells (Bellot et al., 1990) high-affinity binding sites showed an elevated rate of association. Sample data sets are shown in Fig. 2. The fraction of added EGF that was bound as a function of time is plotted. For a single rate of association the initial slopes of the curves would be the same, that is the rate of association would be proportional to EGF concentration. However, for untreated A431 cells (Fig. 2 *a*) a higher rate of association is seen at lower EGF concentrations. Treatment of these cells with PMA which specifically inhibited high-affinity binding for an equilibrium binding experiment (data not shown) reduced the rate of association at the lower concentrations of EGF to that seen at highest concentrations, so that initial sections of the curves superimposed (Fig. 2 *b*). For the truncated receptors which show no high-affinity binding with or without treatment, the association rate was constant over the entire range of EGF concentrations tested (Fig. 2 *c*), and was not affected by PMA treatment (Fig. 2 *d*).

Table I lists the values of fitted parameters for the association data. Data were fit to a simple kinetics model presented in the legend to Table I. Data for the HER14 cells have been presented before (Bellot et al., 1990) and are listed in the table for comparison. Binding to the A431 cells which express ~2 million wild type EGFRs per cell, was similar in all respects to that for the HER14 cells, expressing 300,000 receptors per cell. The data for binding to untreated cells were best fit by equations providing for two populations of receptor, high- and low-affinity, $[R]_{hi}$ and $[R]_{lo}$, each with a separate rate of EGF association, $k_{a,hi}$ and $k_{a,lo}$, but with the same rate of dissociation, k_d. Inclusion of terms for the second population, $[R]_{hi}$ and $k_{a,hi}$, decreased the chisquared values for untreated HER14 and A431 cells by 3.6- and 3.1-fold. By F test (Bevington, 1969) there was a probability of less than 0.01 and 0.02 that the improvement of the fit was random for the HER14 cells and A431 cells, respectively.

Treatment of A431 cells with PMA (Fig. 2 *b*) or incubation with monoclonal anti-EGFR antibody, mAb108 (data not shown) reduced the binding curves to EGF binding that scaled with EGF concentration. The same effect of these treatments was seen for the HER14 cells as reported (Bellot et al., (1990), and first four lines of Table I). These data were fit with three parameters, including only one population of receptor. Inclusion of a second population with a different rate of association reduced the chi squared values by 10 to 20% only, with a probability of 0.5 that improvement of the fit was random.

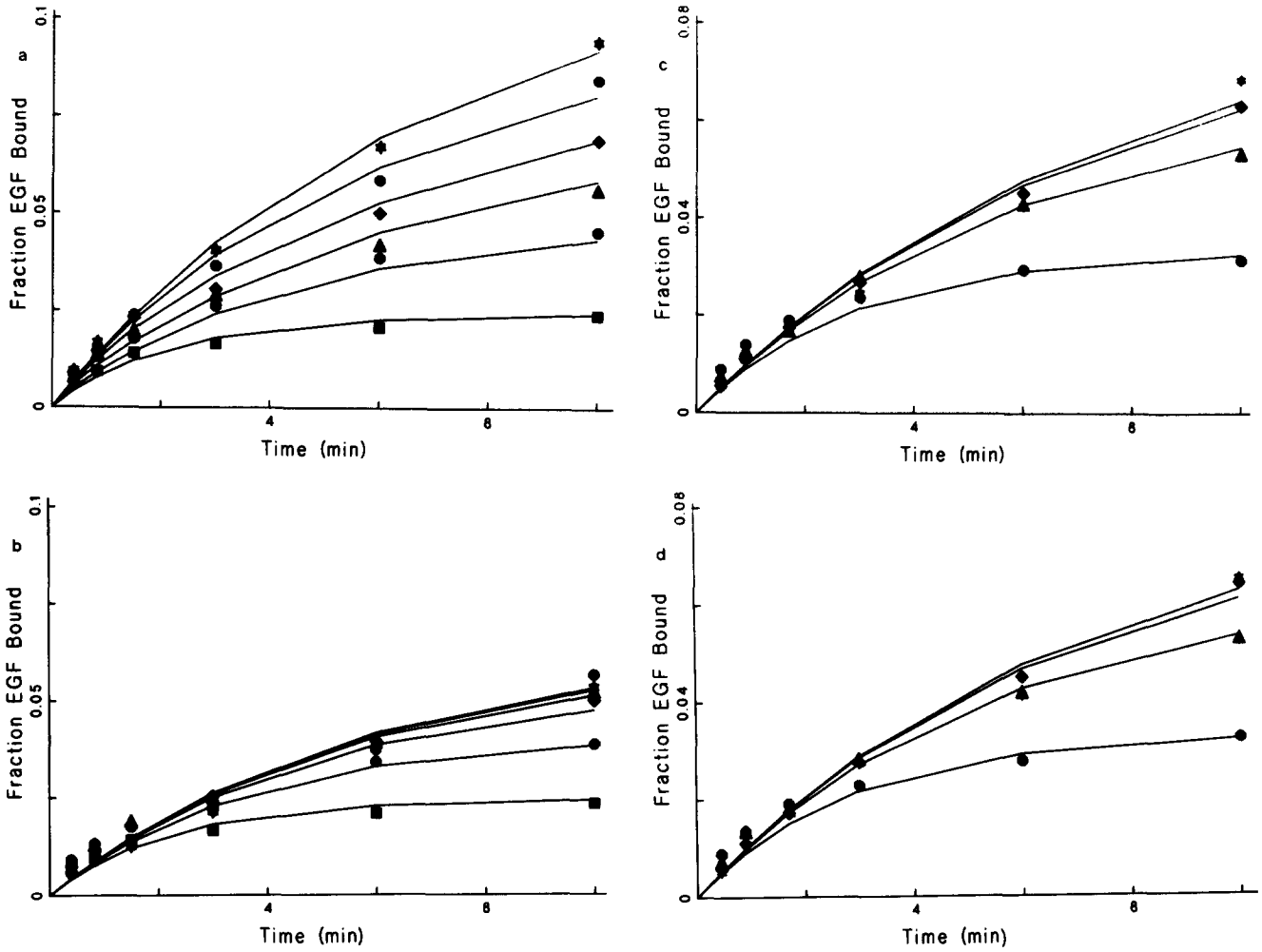

Figure 2. Rate of EGF association. The fraction of added ^{125}I-EGF specifically bound as a function of time is plotted for A431s (*a* and *b*) or for CD533s (*c* and *d*). Cells were either not pretreated (*a* and *c*) or were pretreated with 100 nM PMA (*b* and *d*). Concentrations of EGF added for *a* and *b* were 0.11 (✱), 0.33 (●), 1.0 (◆), 3.0 (▲), 9.0 (●, bottom), and 27 nM (■). In *c* and *d* concentrations added were 0.1 (*), 0.5 (◆), 2.5 (▲), and 12.5 nM (●). Lines drawn present the fitted curves for non-linear least squares fitting to the equation presented in Table 1 solved by numerical integration. Uncertainties of data points ranged from 10% for the 0.1 nM curves to 5% for the higher concentrations.

For the CD533 cells, consistent with the appearance of the binding curves (Fig. 2, *c* and *d*), data for both treated and untreated cells were well fit assuming a single population of receptors, that is, fits were not improved by addition of the second population. Fits to data for all cells and conditions were not improved by allowing for more than one off rate constant. The apparent dissociation constants, K_d (the ratio of the dissociation and association rate constants) and relative numbers of high- and low-affinity receptors agreed well with values estimated by equilibrium binding measurements. For the untreated cells expressing wild type receptor, ~2% of the receptors were of high affinity with an apparent K_d of 0.1 to 0.3 nM. The low-affinity receptors showed an apparent K_d of 8 to 11 nM. For cells expressing the truncated receptor or for all cells treated with PMA or mAb108 to reduce high-affinity binding, the apparent K_d for the single class of receptor seen was very close to that of the low-affinity class, ranging from 5 to 13 nM. For the A431 cells, in one experiment binding rates were determined in the presence of 20 μM phenylarsine oxide which was sufficient to reduce internalization by 90%. There was little effect on fitted rate constants.

Rates of Endocytosis

We next investigated the rate of internalization of receptor ligand complex as a function of EGF concentration. Data were fit to the set of differential equations outlined in Materials and Methods. These equations included a term for recycling of receptor and bound hormone, because as we have previously shown (Honegger et al., 1987; Felder et al., 1990) kinase negative EGFR recycles following internalization. Fig. 3 shows the internalization of EGF at three different concentrations, for HER14 and K721A cells, with and without monensin. The fitted parameters for these data are listed in the first six lines of Table II. Although much less EGF accumulated inside the K721A cells, the fitting routine estimated that the first order rate constant of endocytosis was only twofold less than that for the wild type HER14 cells.

Table I. Kinetics of EGF Binding

	k_a $M^{-1}s^{-1} \times 10^{-5}$	Receptors/ cell	k_d $s^{-1} \times 10^3$	Apparent K_d nM
HER14				
Control high:	98.1 ± 2.3	7.1×10^3	2.9 ± 0.2	0.30
low:	2.54 ± 0.38	3.9×10^5	2.9 ± 0.2	11.4
PMA treated	3.68 ± 0.42	3.5×10^5	1.9 ± 0.3	5.1
+mAb108	4.56 ± 0.31	3.5×10^5	4.6 ± 0.3	9.6
A431				
Control high:	120 ± 5.6	2.1×10^4	1.6 ± 0.2	0.13
low:	2.09 ± 0.38	1.8×10^6	1.6 ± 0.2	7.7
PMA treated	2.80 ± 0.42	1.9×10^6	1.7 ± 0.3	9.4
+mAb108	1.83 ± 0.31	2.2×10^6	2.7 ± 0.3	12.7
CD533 (truncation)				
Control	2.94 ± 0.38	1.4×10^6	2.0 ± 0.2	6.7
PMA treated	2.97 ± 0.42	1.5×10^6	2.2 ± 0.3	7.5

Listed are the best estimates of values of the parameters fit to the equation below. (Note that "Receptors/cell" represents fitted value for the estimated number of receptors per cell, and does not refer to receptor occupancy.) Uncertainties listed were estimated by the fitting procedures. The fitted values for untreated A431s and HER14s were after fitting for two on rates. The remaining samples were after fitting to one on rate. Fitting to two on rates for the latter did not improve the fit. Equation:

$$\frac{d[E:R]}{dt} = \frac{d[E:R]_{hi}}{dt} + \frac{d[E:R]_{lo}}{dt}$$

$$= k_{a,hi}\,[EGF]\,[R]_{hi} + k_{a,lo}\,[EGF]\,[R]_{lo} - k_d\,([E:R]_{hi} + [E:R]_{lo})$$

where

$[E:R]_{hi}$, $[E:R]_{lo}$ = concentrations of occupied high- and low-affinity receptors, respectively
* $[R]_{hi}$, $*[R]_{lo}$ = concentrations of unoccupied high- and low-affinity receptors, respectively
* $k_{a,hi}$, $*k_{a,lo}$ = on-rate constants for high- and low-affinity receptors, respectively
* k_d = rate constant of dissociation (assumed the same for high- and low-affinity receptors.

* Fitted values.

The data taken after monensin treatment support this result. Monensin has been shown to inhibit recycling for many different receptors and cell types, and has not been reported to effect internalization. With monensin, the rate of accumulation of EGF inside the wild type cells was not changed, suggesting that at least in the early time course these cells do not recycle EGF or receptor. For the K721A cells with monensin, EGF was accumulated inside much more effectively at all EGF concentrations, very nearly as fast as for the HER14 cells. The values for the endocytotic rate constant, k_e, reported by the fitting procedure were not changed by the monensin treatment. Further note that the rate of internalization for the truncated EGF receptor, CD533, was 60-fold lower than for the kinase negative K721A cells, and the fitted value for k_e (Table II) and the amount of accumulation of internal EGF for these cells (data not shown) did not change, suggesting further that for these cell lines monensin does not enhance membrane turnover.

The results for the remaining cell lines are presented in Table II. Experiments for all cell lines were repeated in the presence of monensin as well, with no affect on the amount of EGF accumulated, on the fitted values of k_e or on the apparent first order rate constant of recycling, k_{ex}, with one other exception. For cells expressing wild type receptor, both A431 and HER14 cells, pretreatment with PMA

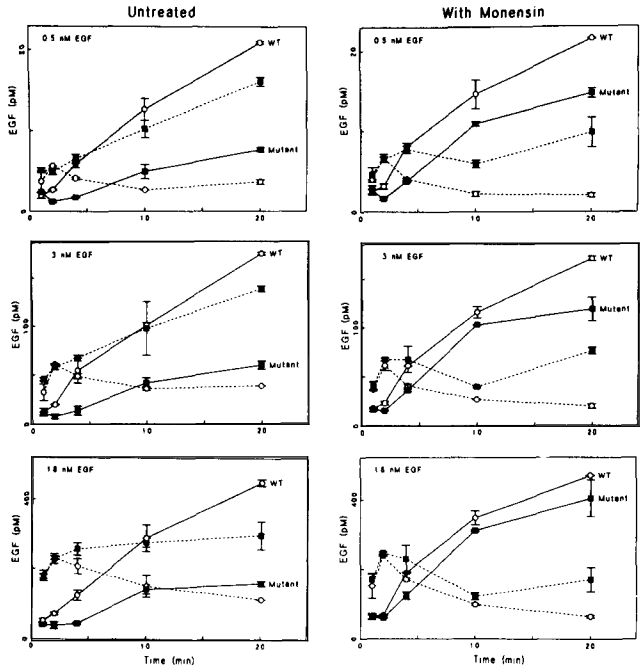

Figure 3. Internalization of ^{125}I-EGF. Plotted are surface bound (– – –) and internalized (———) ^{125}I-EGF for cells expressing wild type (○) and mutant kinase negative receptor (■). To cells at 37°C, 0.5, 3, or 18 nM EGF was added at time "0" to top, middle, and bottom panels, respectively. Cells in the panels on the right were pretreated for 20 min at 37°C with (and tested in the continued presence of) 50 μM monensin.

Table II. Rate Constants of Internalization and Recycling

		No Monensin		With Monensin	
	[EGF]	k_e*	k_{ex}*	k_e*	k_{ex}‡
Her14	0.5 nM	0.47	ns	0.50	0.01
	3 nM	0.29	0.05	0.16	ns
	18 nM	0.20	ns	0.22	ns
K721A	0.5 nM	0.15	0.34	0.16	0.01
	3 nM	0.15	0.42	0.18	ns
	18 nM	0.12	0.15	0.14	ns
Her14, PMA-Treated	0.5 nM	0.24	0.02	0.23	0.01
	3 nM	0.20	ns	0.20	ns
	18 nM	0.12	0.16	0.26	0.03
CD533	0.5 nM	0.004	–§	0.003	–
	3 nM	0.004	–	0.004	–
	18 nM	0.003	–	0.003	–

Rate constants of internalization and recycling for untreated HER14 cells ("wild type" receptor), K721A cells (kinase negative receptor), and HER14 cells pretreated for 20 min with 100nM PMA). "With Monensin" values were for data taken on cells that were pretreated with 50 μM monensin for 20 min at 37°C and tested with monensin remaining in the incubation medium. Cells treated with both PMA and monensin, were preincubated first with PMA for 20 min at 37°C, then with monensin and PMA for an additional 20 min. The estimated uncertainty for the listed parameters as calculated by the fitting procedure was always <10% of the values. Each condition was done at least twice. The best-fit values for the parameters for each condition agreed within 20%.

* Apparent first order rate constant of endocytosis, (min^{-1}).
‡ Apparent first order rate constant of recycling, or exocytosis, (min^{-1}).
§ The values for the rate of recycling of CD533 were not well constrained by the protocol or fitting procedure, because so little EGF was internalized during the incubations.

resulted in a reduction of the amount of EGF accumulated inside if high concentrations of EGF were used (Table II, line 9 and Fig. 4). If these cells were treated with 50 μM monensin after PMA treatment (and left in the continued presence of both), the amount of EGF accumulated was increased to the same level as for untreated cells. Hence, from this analysis it appears that PMA pretreatment induced recycling of EGFRs internalized in response to EGF, under conditions of high occupancy. We note that it has been reported that EGFRs internalized in response to PMA alone are recycled (Beguinot et al., 1985). Further, for A431 cells recycling of receptor without PMA treatment has been demonstrated (Sorkin et al., 1989). Further, we note that PMA treatment inactivates the EGFR tyrosine kinase. Since our data show that internalized kinase inactive receptor recycles, the apparent enhancement of recycling after PMA treatment might result from kinase inactivation. K721A receptor internalization and recycling was unaffected by PMA treatment (data not shown). We do not have an explanation for why the effect was not seen at less than 3 nM EGF.

Relationship of Rate of Internalization to Rate of Binding

For the cell lines tested the rate of EGF binding and the rate of EGF internalization are plotted versus EGF concentration in Fig. 5. Cells were allowed to bind and internalize EGF at 37°C as described and were fit to the set of differential equations listed in Materials and Methods. The rate of binding (Fig. 5 *a*) is presented as the apparent first order rate constant for EGF binding, which represents an average rate of binding, without the complication of separating rates for high and low affinity. Similarly the rate of internalization curves (Fig. 5 *b*) display the "averaged" apparent first order rate constant for EGF internalization. The HER14 cells, the K721A kinase mutant cells, and the T654Y cells all displayed high-affinity binding by equilibrium analysis, and all showed a higher average rate of binding at low EGF concentrations. Upon treatment of the HER14 cells with PMA or with binding of mAb108, the elevated rate of binding at low EGF was not seen, instead the average rate of binding was independent of EGF concentration and was the same as the rate for untreated HER14 cells at the highest EGF concentration. In agreement with the data presented above, the rate of binding for the CD533 truncation mutant was similarly independent of EGF concentration and was the same as the lower rate for saturated wild type receptors.

For the HER14 cells, the rate of internalization was elevated at lowest EGF concentrations, but the elevation was inhibited by PMA or mAb108 treatment. Both treatments also inhibited the elevated rate of binding. The T654Y and

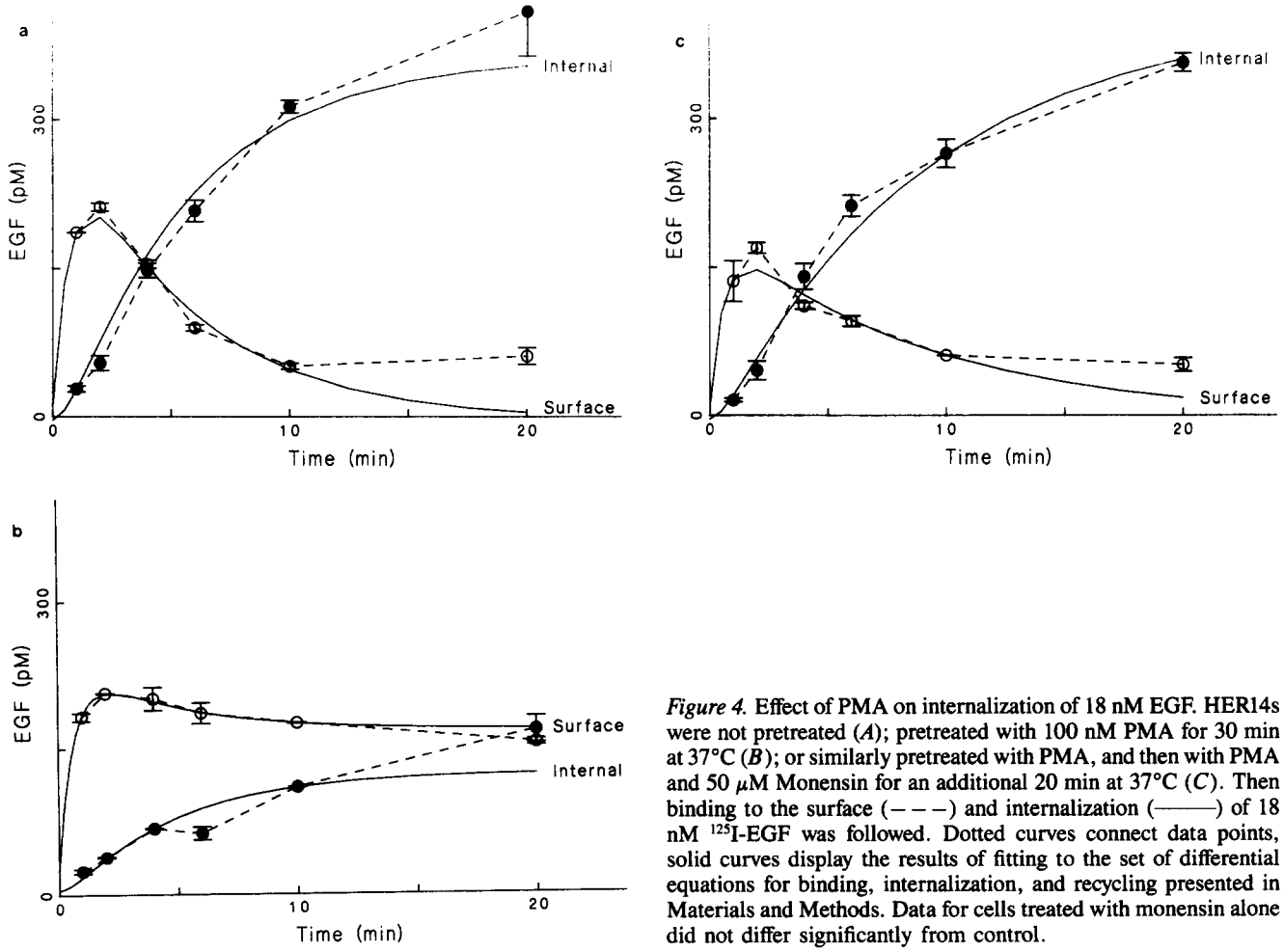

Figure 4. Effect of PMA on internalization of 18 nM EGF. HER14s were not pretreated (*A*); pretreated with 100 nM PMA for 30 min at 37°C (*B*); or similarly pretreated with PMA, and then with PMA and 50 μM Monensin for an additional 20 min at 37°C (*C*). Then binding to the surface (– – –) and internalization (———) of 18 nM ^{125}I-EGF was followed. Dotted curves connect data points, solid curves display the results of fitting to the set of differential equations for binding, internalization, and recycling presented in Materials and Methods. Data for cells treated with monensin alone did not differ significantly from control.

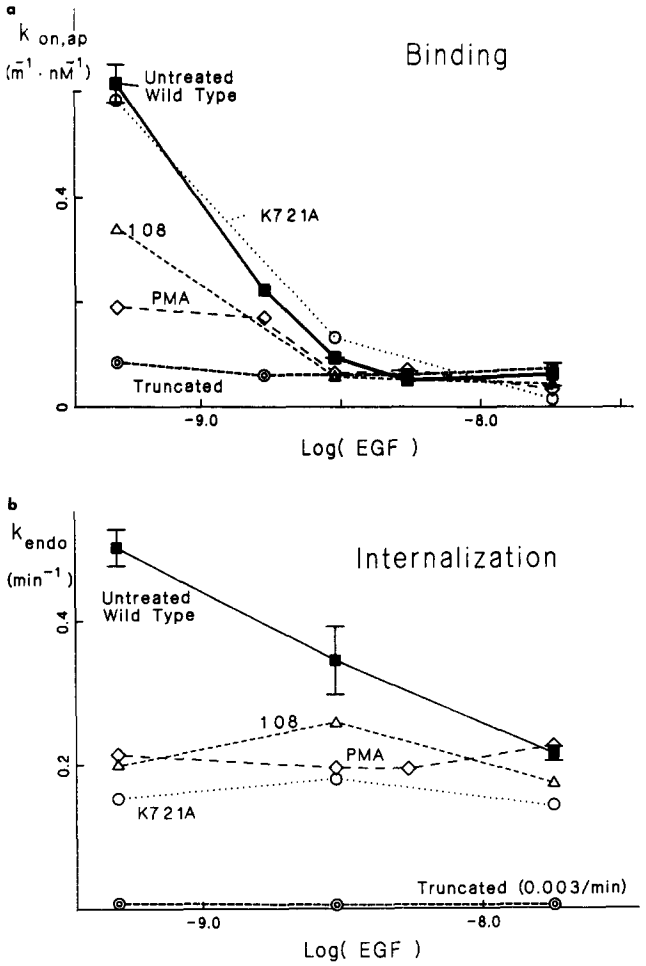

Figure 5. Comparison of rates of binding and endocytosis. Internalization data were fit according to the equations listed in Materials and Methods, assuming a single population of receptor. The single rate constants for binding, labeled $k_{on,ap}$, each representing an averaged rate of association of EGF with the cells, are plotted in Binding panel as functions of EGF concentration. Untreated wild type cells, HER14s, and untreated cells expressing kinase negative point mutant receptor (*K721A*) clearly showed elevated averaged rates of association at low EGF concentrations. CD533 cells (*Truncated*), and phorbol-treated HER14s (*PMA*) showed rates of binding independent of EGF concentration. (PMA treatment did not affect the rates of internalization or recycling for K721A, data not shown.) HER14s in the presence of 100 nM mAb108 (*108*) showed significantly less elevation of binding at low EGF. For untreated wild type cells, HER14s, the best estimate of the fitted parameters from three separate experiments were averaged together and drawn with standard deviation of the result (■). For the remaining cells and conditions, representative curves were plotted. The fitting equations are listed in Table 2. (*Internalization Panel*) For the same set of experiments as in "Binding" panel, the estimated first order rate constant of internalization k_{endo}, for the cells and conditions listed for binding panel are similarly plotted against EGF concentration. Only untreated wild type HER14 cells showed a concentration-dependent rate of ligand internalization.

K721A cell lines showed no elevated rate of internalization even though they both displayed high-affinity, elevated rate of binding. At all concentrations the rate of internalization of the truncation mutant, CD533, was 60-fold lower than that for any other of the EGF receptors studied.

Discussion

Elevated Rate of Binding of High-affinity Receptors

We have shown that with three very different methods for inhibiting high-affinity EGF binding to intact cells, there is no measurable effect on the rate of dissociation of EGF at 4°C, but there is a decrease in the rate of association of EGF for low concentrations. The following conditions were compared: truncation of the cytoplasmic domain, treatment of cells with PMA, or binding of a mAb, mAb108. All of these treatments had no effect on the rate of EGF association with cells at saturating EGF concentrations, but reduced the average initial rate of binding by twofold at 0.1 nM EGF. These data are most consistent with a model which assumes two populations of receptor, one high-affinity and one low-affinity with the high-affinity population making up 1 to 2% of the total and differing from the low-affinity population only in on rate. A recent study with a mAb, mAb2E9, in part, supports this notion (Berkers et al., 1991). mAb2E9 appears to reduce low-affinity EGF binding, but not high-affinity binding in a complementary fashion to mAb108 (DeFize et al., 1990). In the presence of mAb2E9, the rate of EGF binding to cells as measured at 4°C was increased, while with PMA treatment the rate was decreased. The kinetics model used, however, suggested a more complicated conclusion, with effects on both the association and dissociation rate constants.

We note that the off-rate measurements were performed at 4°C, while measurement of association kinetics and internalization kinetics were performed at 37°C. Off rates can be estimated at 37°C, but at that temperature the rates of dissociation and internalization are very similar (Waters et al., 1990), and therefore difficult to separate analytically. Because our different receptors internalized ligand at different rates, measurement of off rates at 37°C would have been problematic. Despite this, we feel confident in our general conclusions that differences occurred only in rates of association and internalization for two reasons. We have seen no evidence for differences in dissociation rates at 4°C. In addition, we saw no improvement in goodness of fit measures for the 37°C experiments of internalization or association when fitting equations included terms for multiple rates of dissociation.

Surface Effects

Surface effects could be important in defining the true rates of reaction of EGF with receptor, since the receptor is highly concentrated and confined to a surface. Theoretical analyses of interactions of this kind have been developed, and predict that the confinement of more than a few thousand receptors to a cell surface may make the surface-averaged rate of ligand association independent of receptor number, and may make the apparent rate of ligand dissociation a function of receptor occupancy (DeLisi and Wiegel, 1981). The effects for observed on rates and off rates result when diffusion to or away from the surface becomes rate limiting instead of the actual processes of attachment or detachment, that is when the on rate approaches the "diffusion limit," roughly 1×10^9 M/s. If surface effects are important in our case, then the observed apparent rate of association with the cells would be highest at low ligand concentrations. Coupled with

this, the observed rate of dissociation from the surface would be highest when receptors are completely occupied, and rates of dissociation (below maximal occupancy) would be elevated by addition of unlabeled, competing ligand. However, the problem of diffusion limited surface effects cannot be critical to our conclusions here for these reasons. The nonlinear Scatchard curve seen for wild type EGFR cannot in the first place result directly from these effects. While the kinetics of both association and dissociation are altered, the equilibrium binding is not (DeLisi and Wiegel, 1981). Secondly, if the association rate we observed is independent of receptor number, then the apparent on rate constant (on a per receptor basis) for the A431 cells should have been three- to fourfold less than for the HER14s, since the A431s express three- to fourfold more receptor; yet the on rates were the same. In addition, the conditions used which reduced high affinity binding (PMA or mAb108 treatment or cytoplasmic domain truncation) did not reduce receptor number, yet the association rates were decreased at low EGF. Hence the elevation in rate of EGF binding at low EGF, and the inhibition of the elevation appear to be independent of diffusion limited, surface effects. It is important to note, however, that the rate of EGF dissociation was enhanced by unlabeled EGF roughly fivefold for all conditions tested. We note, therefore, that our estimates of binding rate constants apply to the conditions used and the method of analysis, and probably reflect processes involving more steps than simple attachment and detachment.

Trafficking of Kinase Negative Point Mutant Receptor

Two articles have appeared (Glenney et al., 1988; Chen et al., 1989) which suggest that the intrinsic kinase activity of the EGF receptor (EGR-R) is required for endocytosis. These studies were in contradiction to two reports from our group (Honegger et al., 1987; Felder et al., 1990). The data presented here (Fig. 3) support our previous conclusions that endocytosis of the kinase negative receptor is rapid and efficient, and that inactivation of the kinase most importantly increases the rate of receptor/ligand recycling. We have fitted internalization data to a model allowing for recycling, for untreated cells and for cells treated with monensin. Monensin has been widely used to test for recycling of receptors. It has been shown to inhibit recycling of several receptors including receptors for LDL (Basu et al., 1981), insulin (Whittaker et al., 1986), transferrin (Stein et al. 1984), ASGP (Berg et al., 1983), and EGF in hepatocytes (Gladhaug and Christoffersen, 1988). It has effects on cells aside from blocking exocytosis, but it does not appear to affect the internalization phase of receptor trafficking. It had no effect on the trafficking of the wild type EGF receptor, in HER14, or, importantly, on the trafficking of the poorly internalized truncation mutant, CD533. Further, we have shown that monensin inhibits recycling without affecting internalization of the transferrin receptor in these same cell lines (Felder et al., 1990)

At all concentrations tested wild type receptor was accumulated inside the cells faster than kinase negative receptor, but with monensin treatment, the difference was reduced. Monensin had little effect on the wild type receptor, but increased intracellular accumulation of kinase negative receptor roughly twofold at all EGF concentrations. At highest EGF concentrations, monensin completely reduced the difference between wild type and kinase negative receptor. At lowest EGF concentrations, however, the difference was still apparent (discussed below).

We conclude that kinase negative mutant receptor is rapidly and efficiently internalized; the specific rate of internalization of this receptor appears to be nearly the same as for normal receptor. The major difference between normal and kinase negative receptor, lies in the rate of recycling, which is 10-fold elevated for the mutant. It is noteworthy that these are apparent rate constants which depend upon the model used. A more complete kinetics model will improve further upon these estimates. In support of the conclusions from kinetics considerations are the following additional experimental data: (*a*) Similar to the effects of monensin treatment, for cells internalizing ligand at 20°C (which inhibits degradation and slows recycling) internalization and surface down regulation kinetics are similar for wild type and mutant receptor (Felder et al., 1990); (*b*) EGF is degraded to TCA soluble form with similar kinetics by cells expressing wild type or mutant receptor (Honegger et al., 1987). The degradation is saturable and inhibited by lysosomal enzyme inhibitors. (Hence the rate of recycling of EGF by kinase negative cells does not significantly slow the kinetics of EGF degradation. Apparently enough internalized EGF is sequestered for degradation at each round of the recycling pathway to not be rate limiting for the much slower rate of degradation); (*c*) Internalization of the mutant receptor itself can be demonstrated using radiolabeled anti-EGFR antibodies (Felder et al., 1990) and by immunogold labeling of cryosections; (*d*) The morphological pathway of internalization for kinase negative receptor identified using EGF peroxidase and anti-EGFR-gold conjugates is indistinguishable from normal receptor during the early stages of endocytosis (Felder et al., 1990).

Recent studies of the trafficking of two related tyrosine kinase receptors, the PDGF and CSF-1 receptors, are consistent with our results. A point mutant PDGF receptor is internalized twofold more slowly than is the wild type receptor (Sorkin et al., 1991*a*). Similarly, a point mutant CSF-1 (c-fms) receptor is internalized at very nearly the same rate as is wild type (Carlberg et al., 1991). In the former study it was not directly tested whether the point mutant PDGF receptor was recycled. In the latter study clear recycling of the kinase negative CSF-1 receptor (but not wild type) was demonstrated.

Elevated Internalization at Low EGF Concentrations

We propose that high-affinity receptors are internalized more quickly than are low-affinity receptors, because they are "preactivated," that is, they have already completed an activation step for endocytosis which is normally rate limiting for this receptor. This step may involve a conformational change, dimerization, or aggregation into coated pits, for example. In this model, all EGFR is internalized by the same pathway but low-affinity receptors, after EGF binding, would first have to complete this activation step.

We propose the following model to interpret the results presented that EGF induces the internalization of EGFR, and that wild type receptor internalizes more quickly at low EGF concentrations. The EGFR is pictured as existing in two states at equilibrium, R_{hi} and R_{lo}. R_{hi} has a higher affinity for EGF and for some element of the endocytic apparatus. EGF binds with higher affinity to R_{hi}, and so by

thermodynamic considerations must shift the equilibrium toward the R_{hi} state. We propose that this shift in equilibrium toward R_{hi} is strong enough and fast enough so that most occupied receptor is converted to the "R_{hi}" state. (Since as our data suggests affinity to EGF is determined by on rate, conversion of R_{lo} to R_{hi} for occupied EGFR is not reflected in measured affinity. Rather, the estimate from equilibrium measurements that 2% of receptor is high-affinity reflects the unoccupied condition). EGF binding, then, increases the affinity of most of the surface receptor for some element of the endocytic apparatus and induces internalization. For example, high-affinity receptors may result from a conformation stabilized by aggregation of receptor into coated pits. Receptors already aggregated bind EGF more quickly, and are internalized more quickly. Receptors disperse on the cell surface bind EGF 50-fold more slowly, and then must diffuse to or assemble within the coated pits before being internalized.

The model is consistent with our data. Whatever method was used to inhibit high-affinity EGF binding, also reduced the rate of internalization at low concentrations of EGF. However, the converse clearly wasn't true, two mutants for which high affinity binding remained showed no elevation of internalization at low EGF. We suggest, then, that high affinity binding is necessary but not sufficient for nor dependent upon the preactivation step. Also necessary are a functional kinase domain and the submembrane Thr654 site. For the tyrosine kinase function, our data do not test whether autophosphorylation or phosphorylation of a cellular substrate is necessary; however, two recent reports suggest that EGFR missing most of the carboxy terminal autophosphorylation sites are also slightly more slowly internalized (Sorkin et al., 1991*b*; Helin and Beguinot 1991), suggesting that autophosphorylation is important. For the protein kinase C Thr654 site, it is not clear whether phosphorylation of the site or the structure of the receptor at this site, very near the plasma membrane is important. When wild type cells were treated with PMA to down regulate protein kinase C (or at least to decrease phosphorylation of proteins in response to subsequent PMA treatment) no effects were seen on the kinetics of binding or internalization (data not shown), suggesting that phosphorylation is not necessary.

An alternative model has been presented to interpret similar kinds of experiments performed on a different cell background (Chen et al., 1989; Lund et al., 1990*a*). The model suggests that EGFR is internalized fairly rapidly in the absence of EGF, at 0.05–0.1/min. The model states that EGF induces the internalization of EGFR at a faster rate, 0.2–0.4/min, but that this induced pathway is physically distinct from the uninduced pathway, and is limited in capacity to roughly 10,000 to 50,000 receptors/cell. We do not believe this a plausible model for three reasons. First, the capacity for fibroblasts to internalize transferrin receptor through coated pits is very large. A rate of 0.4/min is maintained even when the receptor is overexpressed to 500,000 receptors/cell (Jing and Trowbridge, 1990). Hence, the apparatus for internalization itself must not be limiting. Second, saturating (100 nM) EGF induces rapid redistribution to and internalization through coated and noncoated pits for a large majority of its receptor on A431 cells expressing 2 to 3 million receptors/cell, as visualized by EM (Haigler et al., 1979). This was an important result clearly discriminating the characteristics of endocytosis for the EGF receptor from that of the LDL receptor. For LDL and transferrin receptors, the surface distribution of receptor appears the same whether receptor is labeled at 4°C or is incubated with labeled ligand at 37°C for some time and then fixed. Third, the internalization of unoccupied EGFR appears to be very slow: the truncated receptor, CD533, is internalized at 0.003/min (as reported here), and with treatment of HER14s with 50 μM monensin unoccupied (constitutively recycling) transferrin receptors were rapidly down regulated, while unoccupied surface EGFR decreased with an apparent first order rate constant of 0.002/min (our unpublished results). Hence our data, coupled with other results argue against this model.

We note that some of our data appear to be in conflict with results reported in Chen et al. (1989), Lund et al. (1990*a*, *b*), and Wiley et al. (1991). Most data are similar qualitatively, however three important differences exist: (*a*) Our estimates for k_e are higher, ranging from 0.5/min down to 0.2/min for the wild type receptor at low and high EGF, respectively, while their estimates range from 0.3 down to 0.05/min. At the same time, our truncated EGFR, CD533 having only nine cytoplasmic amino acid residues, shows a much lower rate of internalization, 0.003/min, than they have reported for a truncated EGFR having only two residues inside the cells, of 0.05/min. If the internalization rates for the truncated receptors could be used as a loose estimate of the rate of internalization of unoccupied wt receptor (as both groups have asserted) then this difference is critical. Using the lower estimate that we report, EGF greatly enhances internalization of overexpressed, fully occupied receptors, and of kinase negative receptors. However, using their higher estimate, a different conclusion could be reached; (*b*) Our results for the point mutant EGFR at Thr654 show that this region is critical to enhanced internalization at low EGF, while the results reported by Lund et al. (1990*b*) suggest that it is unimportant. In our mutant, threonine was replaced with tyrosine, while in theirs it was replaced with alanine; and (*c*) Results for the kinase negative point mutant receptors (K721A in our reports, K721M in theirs) yielded different conclusions. We conclude that kinase negative EGFR is internalized in response to EGF binding normally, but that it does not show enhanced internalization at low EGF as does the wild type receptor. They conclude EGF does not stimulate internalization of kinase negative EGFR. We have reported that monensin doesn't affect internalization of wt EGFR but greatly enhances intracellular accumulation of kinase negative receptor (Felder et al., 1990 and herein). Wiley et al. (1991) report no effect of a lower dose of monensin. Further, we report that recycling of intact EGF back to the medium is much greater for kinase negative receptor. Wiley et al. (1991) also report the same rate of fairly efficient recycling for both wild type and kinase negative receptor. The reasons for these differences are not clear.

We favor the model we have presented, but it should be pointed out that the system may well be more complicated. For example PMA down regulates high-affinity EGF binding by stimulating protein kinase C–mediated phosphorylation of Thr654, and EGF binding stimulates phosphorylation at the same residue. Perhaps at low EGF, for which the rate of occupation of total receptors is relatively slow, unoccupied, high-affinity receptors may be phosphorylated at Thr654,

and down modulated. That pretreatment of cells with EGF can alter subsequent EGF binding and trafficking has been demonstrated (Kuppuswamy and Pike, 1989). Alternatively, EGF binding at low occupancy might recruit unoccupied receptor for internalization along with occupied receptor, that is, the stoichiometry of internalization or even of binding may change with occupancy. These models may also be consistent with the data reported here. Further mutational analysis and kinetics experiments on cell surface receptor and on solubilized ligand binding domains may be helpful in testing these possibilities.

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