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ARTICLES

pH Sensitivity of Epidermal Growth Factor Receptor Complexes

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Abstract The association/dissociation binding kinetics of ^{125}I-labeled mouse epidermal growth factor (EGF) to receptors on human fibroblast cells in monolayer culture have been measured at 4°C as a function of extracellular pH from pH 5–9. At pH 8, steady-state total binding is maximal. As pH is lowered to 6.5, total binding monotonically decreases dramatically. It changes further only slightly between pH 6.5 and 5 to about 20% of the maximum binding value. Scatchard binding plots at pH 7.5 and above show the commonly observed concave-upward, non-linear curve; as pH is lowered, this plot becomes much more linear, indicating that the "high affinity" bound receptor population is greatly diminished. Application of our ternary complex binding model [Mayo et al., J Biol Chem 264:17838–17844, 1989], which hypothesizes complexation of the EGF-bound receptor with a cell surface interaction molecule, indicates that pH may have some direct effects on ternary complex formation, but the major effect is on EGF-receptor dissociation.

Key words: binding kinetics, epidermal growth factor, fibroblast cells

Binding of the protein hormone epidermal growth factor (EGF) to cellular transmembrane receptors [Carpenter and Cohen, 1976] triggers a series of physiological events that ultimately lead to increased RNA, DNA, and protein synthesis and cell division [Carpenter and Cohen, 1979]. Since the initial step in the biological action of EGF is cell surface receptor binding, considerable scientific interest has been focused on this event. Parameters for ligand binding to the EGF receptor are most frequently derived from the concentration dependence of equilibrium binding data which have been transformed into a Scatchard plot [Scatchard, 1949] using a two-state binding model. Mechanistic aspects of EGF-receptor binding and trafficking, however, can only be derived from knowledge of the ligand binding and trafficking reaction rates. The rates at which EGF-receptor association and dissociation reactions occur under various conditions, for example, are critical to an understanding of cell surface and cytoplasmic dynamic processes.

Previously, we have introduced a ternary complex binding model to more fully explain EGF-receptor kinetic and steady-state binding data [Mayo et al., 1989]. The so-called high affinity class of EGF receptors which showed themselves in non-linear Scatchard plots could be explained by our model when they were equated with the EGF-bound receptor associating with a cell surface interaction molecule, perhaps a coated pit protein. In other receptor binding systems, various three-state models have also been used to account for inadequacies in fitting ligand-receptor binding data with a two-state model. Such studies have generally concluded that receptor-ligand complexes, once formed, further associate with a membrane interaction molecule (i.e., another protein) [Jacob and Cuatrecasas, 1976; De Lean et al., 1980; Adamson and Rees, 1981] thereby imparting different binding affinities.

Many ligands which bind to cell surface receptors are rapidly internalized by receptor-mediated endocytosis [Carpenter, 1987]. Typically,

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endocytotic vesicles containing ligands become acidified to a pH of about 5–5.5 within the first few minutes after internalization [Tycko and Maxfield, 1982; van Renswoude et al., 1982; Tycko et al., 1983]. Ligand receptor complexes respond in various ways to acidification. Several ligands, including EGF [Haigler et al., 1980], insulin [Posner et al., 1977], α_2-macroglobulin [Maxfield, 1982], asialoglycoproteins [Ashwell and Morell, 1974], and lysosomal enzymes [Gonzalez-Noriega et al., 1980] dissociate from their receptors. For ligands such as these which dissociate from their receptors at low pH, it is unclear whether the pH dependence is a property of the ligand, the receptor, the membrane, or what.

In this report, kinetic (association and dissociation) and equilibrium ^{125}I-EGF-receptor binding experiments were performed as a function of extracellular pH from pH 5–9. Human foreskin fibroblast cells were used as the model system, and binding experiments were done at 4°C in order to suppress receptor internalization. As the pH is lowered from 8 to about 6, overall ^{125}I-EGF-receptor binding is attenuated about fivefold. Results from association and dissociation kinetic plots indicate that this commonly observed pH dependence in apparent EGF-receptor binding rests mostly in EGF-receptor dissociation.

METHODS

Isolation of EGF

Murine EGF was isolated from submaxillary gland homogenates of adult male albino mice as described by Savage and Cohen [1972], with minor modifications. EGF samples were then further purified of N-terminal cleavage products by HPLC methods discussed elsewhere [Mayo and Burke, 1987]. Purity was checked by gel electrophoresis, amino acid analysis, and proton-NMR. Only α-EGF from the mouse was used in these studies.

Radioiodination of EGF

Iodination of EGF was carried out by a modification [Moriarity and Savage, 1980] of the chloramine-T method [Hunter and Greenwood, 1962]. This method yields ^{125}I-EGF specific radioactivities of 100–300 μCi/μg. Radioactivity was measured in a Packard Gamma Scintillation Counter with a counting efficiency of 73%.

Studies With Fibroblasts

Human fibroblast monolayer cultures were derived from normal male skin biopsies or from frozen foreskin cells purchased from American Type Culture Collection. These cells were routinely propagated in 75 cm^2 plastic T flasks [Hollenberg and Gregory, 1977; Hollenberg and Cuatrecasas, 1973] in anti-biotic-free Dulbecco's modified Eagle medium, with Earle's balanced salts supplemented with 10% (v/v) fetal bovine serum, 0.5% (v/v) Nystatin, 1.0% (v/v) penicillin streptomycin, and $NaHCO_3$. Cells were subcultured as 1.5 cm diameter monolayers in 24-well multiwell trays. After subculture from T flasks into multiwell trays, monolayers were refed five times at two day intervals with growth medium containing 10% (v/v) serum and were used within 7–10 days after reaching confluency.

All binding experiments were performed at 4°C where receptor internalization and EGF degradation are considered to be greatly attenuated, if not eliminated. Measurements of the binding of ^{125}I-labeled EGF were performed in the multiwell trays. Prior to the addition of labeled material, cells were washed with MEM, then preincubated for 2 h at the desired pH value in serum-free minimum essential medium supplemented with 0.2% (w/v) Bovine Serum Albumin and 25 mM HEPES. Following addition of ^{125}I-EGF to the cells, the time course of binding was followed; at given time intervals, duplicate or triplicate mini-wells were washed three times with ice-cold albumin-free 0.1 M phosphate buffer and 0.9% NaCl, solubilized for at least 30 min. in 2.5 M NaOH, and the radioactivity measured by scintillation counting.

For ^{125}I-EGF-receptor dissociation kinetics, cells were as usual incubated at 4°C in the presence of ^{125}I-EGF. At various times before and after reaching equilibrium, cells were washed rapidly with cold buffer and the same volume and concentration of unlabeled EGF added back to the cells. The time dependence of ^{125}I-EGF dissociation from receptors was measured as stated above.

The raw data were corrected for non-specific binding by subtracting from the total counts, the amount of radioactivity remaining with the cells in the presence of an excess (1 μg/mL) of unlabelled EGF [Carpenter, 1987]. A secondary control for non-receptor-bound ^{125}I-EGF was

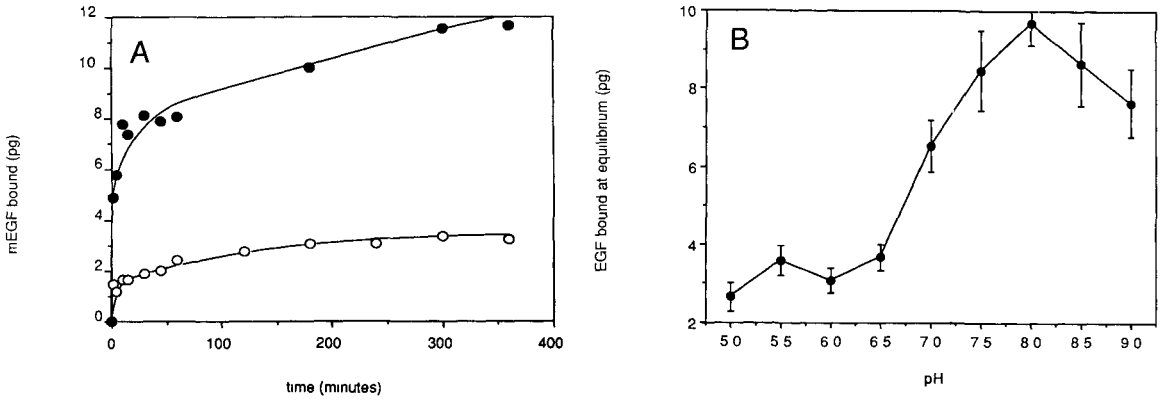


Fig. 1. pH dependence of the time course of ^{125}I-labeled EGF binding Fibroblast cells were plated in 24 miniwells at pH 7 4 and allowed to grow into monolayers as described in the text ^{125}I-EGF at a concentration of 5×10^{-10} M was incubated with 2×10^5 cells in monolayers culture at the desired pH value and at 4°C At various times are after addition of ^{125}I-EGF, wells in duplicate were washed three times with 0 01 M PBS to remove free ^{125}I-EGF, and the cells were solubilized with sodium hydroxide and scintillation counted All values were corrected for non-specific binding These data are shown in (**A**) for pH values 7 5 (●) and 5 (○) Computer fit of the data using the ternary complex model (equation 1) as described in the text yields the solid lines (**B**) gives the equilibrium number of total EGF bound complexes in a more complete pH profile The line drawn through data points is for visual aid only

made by washing cells with 0.1 M acetic acid which is known to dissociate bound EGF from the receptor complex [Matrisian et al., 1984]. Non-specific binding of ^{125}I-EGF accounted for 5–10% of the total counts.

The protein content of monolayers was determined with the Folin-Ciocalteu reagent [Lowry et al., 1951], and the cell number per well was determined by staining trypsinized cells with trypan blue and using a hemocytometer (usually between 150,000 and 200,000 cells per well).

RESULTS

Receptor Binding pH Effect

The kinetics of EGF-receptor binding at low concentrations of ^{125}I-murine-EGF are shown in Figure 1A for pH 7.5 and pH 5.0. The amount of equilibrium binding at pH 5 is roughly a factor of four less than at pH 7.5. This general pH effect on EGF-receptor equilibrium binding has been known for some time [Haigler et al., 1980]. Figure 1b shows a more complete pH dependence of maximum equilibrium binding for a given EGF concentration, 5×10^{-10} M. The trend reflects a titration curve and can be fit with the Henderson-Hasselbalch equation yielding an average pK_a value of about 7.0. While this in itself may be interesting, it says nothing about the molecular nature of the pH effect or about individual reaction steps which could contribute to the effect. Furthermore, since EGF binding kinetics were monitored for 6 h, the possibility of receptor-mediated endocytotic internalization presented itself. To control for this, cells bound with labeled EGF were washed with 0.1 M acetic acid which is known to dissociate surface-bound EGF from the receptor complex [Matrisian et al., 1984]. Counts that remained were either due to internalized EGF or to non-specifically bound EGF. Non-specific EGF binding was controlled for by correcting raw data with that collected from cells that were incubated in the presence of an excess of unlabeled EGF as described in the Methods section. Non-specific binding and/or "internalized" EGF accounted for less than 5–10% of total radioactive counts.

Previously, we introduced a ternary complex EGF-receptor binding model [Mayo et al., 1989] to explain deficiences in the normally employed, simple two-state binding model analysis. That study revealed the necessity for using a generic three-state binding model analysis of kinetic and steady-state EGF-receptor binding data. Symbolically, our model is

$$L_o + R_s \underset{k_r}{\overset{k_f}{\rightleftarrows}} C_s \qquad C_s + P_s \underset{k_u}{\overset{k_c}{\rightleftarrows}} T_s \qquad (1)$$

where L_o is the free ligand, R_s is the free receptor, C_s is the ligand/receptor complex, P_s is the free interaction molecule, and T_s is the ligand/

receptor/interaction molecule complex. k_f and k_r are the rate constants for ligand/receptor binding and dissociation, respectively, and k_c and k_u are the rate constants for complex/interaction molecule association and dissociation, respectively. The other model parameters needing to be determined are R_T and P_T, the total number of surface receptors and cell surface, interaction molecules, respectively. (Some of these parameters are different from those given in Mayo et al. [1989] in order that they be consistent with similar parameters used elsewhere.)

Scatchard Binding Plots

Equilibrium Scatchard plots which display bound/free ^{125}I-EGF as a function of total bound are shown in Figure 2 for data acquired at pH 7.5 and 5. As previously observed by Mayo et al. [1989], the Scatchard plot at pH 7.5 curves upward at higher binding site occupancy. At lower pH, in contrast, the Scatchard plot remains mostly linear, exhibiting a shallower slope. Both data sets yield a total of about $2 \pm 0.3 \times 10^5$ EGF-binding sites (or receptors assuming a 1:1 complex) per cell. While this total number of receptors is, within error, pH independent, the shape of the Scatchard plot is highly pH dependent. The normally observed, higher pH concave-up slope changes smoothly, rather than abruptly, with decreasing pH, indicating a pH-induced transitional change in the system. Optical microscopy indicates that cell morphology (trypan blue staining) was not modified by pH changes between pH 5 and pH 9. Modulation of the high affinity binding component has also been observed in EGF binding studies performed either in the presence of active phorbol esters or in cases where EGF-receptor kinase active/inactive mutant-transfected cells were used [Lund et al., 1990; Wiley et al., 1991].

Since our EGF was radiolabeled by using the chloramine-T method [Hunter and Greenwood, 1962], heterogeneously labelled ^{125}I-EGF may result. Most of our Scatchard plots, for technical reasons, were derived by using a constant amount of radiolabeled EGF and then diluting with various amounts of unlabeled EGF to obtain final experimental concentrations. Such varying specific activities could produce a disproportionate decrease in binding at higher total EGF concentrations if the labeled EGF is not 100% bindable and may give rise to differences as a function of extracellular pH. To explore this possibility, additional Scatchard plots were generated with ^{125}I-EGF of a constant specific activity as was done in Mayo et al. [1989]. Within experimental error, no differences were noted between these data sets, suggesting either that our labeled EGF is homogeneous or that the site of ^{125}I-labeling is removed from the receptor binding site. In addition, both acid wash and excess cold EGF controls were done as described above. These data, therefore, represent cell surface bound EGF.

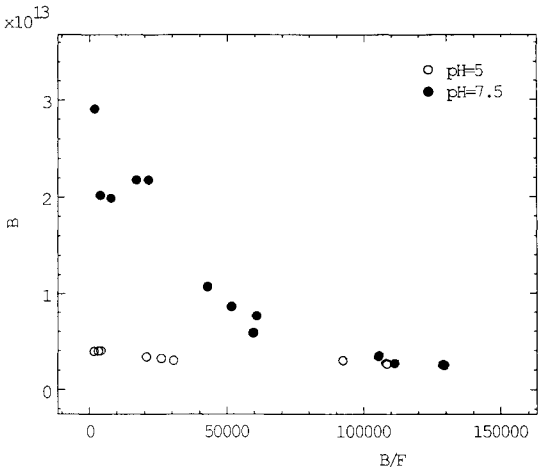


Fig. 2. Scatchard plot of EGF binding. ^{125}I-mouse EGF (2×10^{-11} M) was incubated for 90 min in 0.2 mL wells plated with fibroblast cells in monolayer culture (2×10^5 cells per well) in the presence of increasing concentrations of native, unlabeled mouse EGF. The labeled cells were then washed and treated as described in the text. The bound/free ratio of labelled EGF is plotted as a function of EGF bound to the cells.

Analysis of these equilibrium binding data requires simultaneous solution of model equations at steady-state:

$$dC_s/dT = k_f L_{exog} (R_{tot} - C_s - T_s) - k_r C_s - k_c C_s (P_{tot} - T_s) + k_u T_s \quad (2)$$

$$dT_s/dt = k_c C_s (P_{tot} - T_s) - k_u T_s. \quad (3)$$

These equations assume that the concentration of extracellular ligand (L_{exog} for exogenous) remains constant (i.e., $L_o = L_{exog}$) that the total number of receptor interaction molecules remains constant (no synthesis or degradation) (i.e., $P_{tot} = P_s + T_s$), and that the total number of receptors remains constant (i.e., $R_{tot} = R_s + C_s + T_s$).

Defining $B = C_s + T_s$ as the total number of specifically bound EGF molecules per cell, one

can obtain the following Scatchard plot equation for bound ligand (B) divided by free ligand ($F = L_{exog} - B$) as a function of bound ligand (B):

$$B/F = [2B(R_{tot} - B)]/K_{d1}[(B - P_{tot} - K_{d2}) + [(B - P_{tot} - K_{d2})\,2 + 4BK_{d2}]\,1/2. \quad (4)$$

The unknowns in equation 4 are the total number of receptors (R_{tot}) and the total number of interaction molecules (P_{tot}) as well as K_{d1} and K_{d2}, the equilibrium dissociation constants for binary and ternary complexes, respectively. Each of these parameters can be determined from a separate piece of information from a Scatchard plot. Limit analysis of equation 4 provides approximate formulae useful for interpretation of this plot [Starbuck, 1991]:

$$\text{y-intercept} = R_{tot}P_{tot}/K_{d1}K_{d2}$$

$$\text{initial slope} = -R_{tot}/K_{d1}K_{d2}$$

$$\text{final slope} = 1/K_{d1}$$

$$\text{x-intercept} = R_{tot}.$$

Equilibrium parameters derived from such Scatchard plot analysis indicates that K_{d1} varies the most from 2×10^{-8} at pH 8 to 6×10^{-8} at pH 5. Surprisingly, K_{d2} is essentially invariant over the pH range studied. The ratio of the apparent number of cell surface accessory molecules to the number of cell surface receptors, P_{tot}/R_{tot}, decreases with decreasing pH as shown for example in Figure 3, from about 0.2 at pH 9 to 0.03 at pH 5.

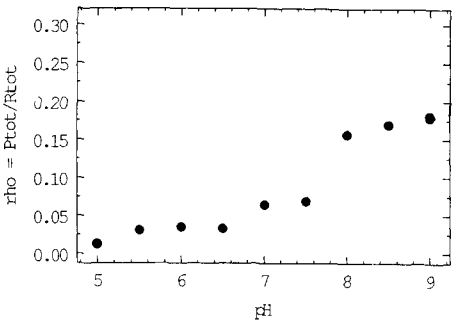


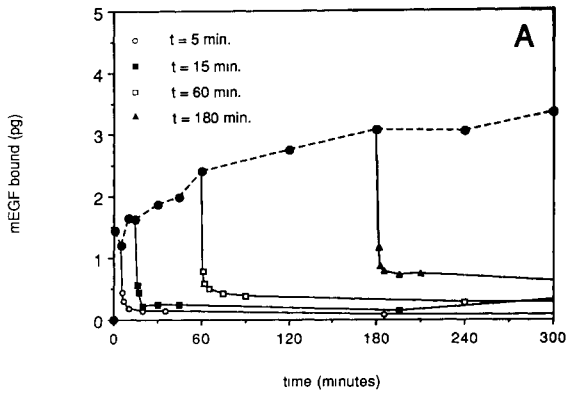
Fig. 3. pH dependence of ternary complex formation. Limit analysis as discussed in the text gives the values of P_{tot}/R_{tot} vs. the extracellular pH from pH 5 to pH 9.

EGF Binding Kinetics

The initial kinetics of the binding reaction of low concentrations of ^{125}I-murine EGF have been measured at pH 7.4 [Mayo et al., 1989] yielding a value for k_f of $1.2 \pm .2 \times 10^6\ M^{-1}\ s^{-1}$. The pH dependence on k_f in this paper has been estimated via analysis of the initial slope of kinetics plots like those shown in Figure 1A. k_f appears to be pH independent with values ranging randomly within the range $1 \times 10^6\ M^{-1}s^{-1}$ to $1.8 \times 10^6\ M^{-1}s^{-1}$. This set of values is consistent with those given in Mayo et al. [1989].

^{125}I-EGF-receptor dissociation binding kinetics data are shown in Figure 4 for pH 7.5 and 5.0. ^{125}I-EGF-receptor dissociation was monitored for several hours with and without the addition of unlabeled-EGF to the buffer. This was done since unlabeled EGF could effect a cellular response to affect ^{125}I-EGF ligand dissociation. The results were the same as might be expected for a simple unimolecular dissociation process. An immediate observation can be made in Figure 4. Biphasic dissociation kinetics are evident at either pH value where dissociation is initially rapid and then slows down dramatically. This effect had been previously noted in Mayo et al. [1989] for data at pH 7.5. What is interesting here, however, is that the separation between the fast and slow dissociation component slopes is more abrupt for the pH 5 data. This in turn suggests that the fast and slow rate constants are farther apart at the lower pH value.

Plotting these dissociation data as natural logarithm of fraction bound vs. time yields Figure 5. Here, dissociation kinetics are shown on a logarithmic scale as a function of ^{125}I-EGF binding or incubation times. An initial "fast" component gives a rate constant of $1.5–2 \times 10^{-2}\ s^{-1}$, while it can be noticed that the "slow" component ordinate intercept varies with increasing incubation time. The slopes of these slow component lines are the same, however, and give a dissociation rate constant of about $3 \times 10^{-5}\ s^{-1}$. While there is sufficient time resolution for determining that the slow component rate constant is pH invariant, there appears to be insufficient time resolution in the fast component to make that same statement. Hence, the safest inference is that differences lie in the fast component. This is consistent with the Scatchard plot analysis given earlier where it was shown that K_{d1} changed significantly with pH, while K_{d2} did



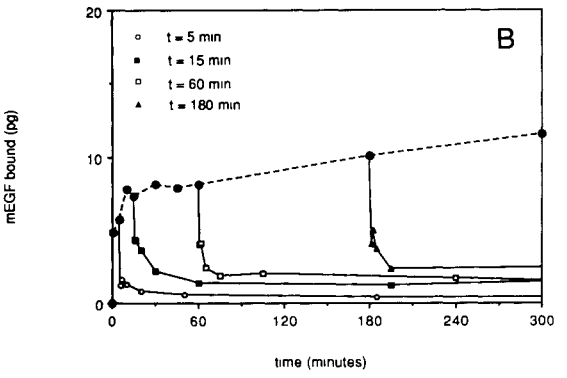
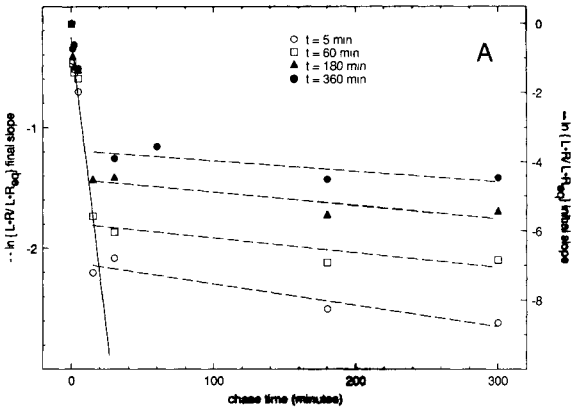


Fig. 4. ^{125}I-EGF-receptor dissociation plots at pH 7.5 and 5.5. At various times during 4°C incubation of cells with ^{125}I-EGF (5×10^{-10} M in 0.2 mL), the binding reaction was halted by two rapid, cold buffer washes, followed by addition of 0.2 mL buffer with or without unlabelled EGF (5×10^{-10} M). The time dependence of counts remaining associated with cells were then monitored as described in Methods. The data have been corrected for non-specific binding and are plotted as the total number of ligand-receptor complex vs. the ^{125}I-EGF incubation time. Times shown indicate time when radioactive ligand was removed. These data are for pH 5 (**A**) and pH 7.5 (**B**).



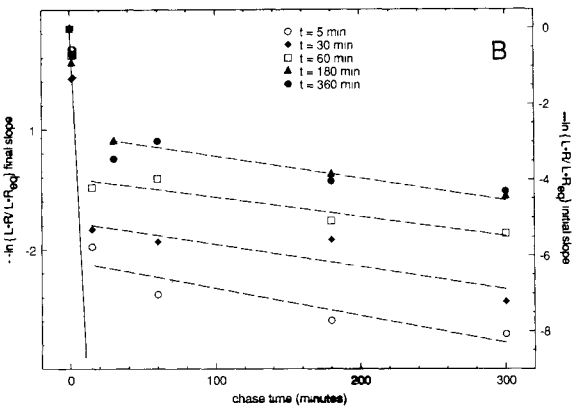


Fig. 5. ln fraction dissociated vs. time. The data in Fig. 4 are shown as the natural logarithm of the fraction ^{125}I-EGF dissociated vs. time for the length of time of ^{125}I-EGF incubation before dissociation kinetics were followed. Solid lines drawn through data points represent least-square fits to the data. Estimated rate constants for fast and slow populations were attained by assuming biphasic dissociation kinetics and deconvoluting the curve as two linear components. The slope of the fraction of fast component dissociated (solid line) yields k_r; the slope of the fraction of slow component dissociated (dashed lines) yields k_u. These data are for pH 5 (**A**) and pH 7.5 (**B**).

not. At pH 5, k_r can be estimated to be about 4×10^{-2} s^{-1} or twice that of k_r observed for pH 7.5. In effect, decreased binding at pH 5 probably lies mostly in the increased dissociation rate. Equilibrium and kinetic values have been summarized in Table I.

In Figure 5, the ordinate intercept varies with time because the populations of the fast and slow components vary. As a function of incubation time, the fast component is decreased in population and the slow component is increased proportionally. If one plots the fractional ordinate intercept of the slow component (from Fig. 5) vs. incubation time, kinetics curves for the formation of ternary complex are obtained for pH 7.5 and 5.0 (Fig. 6). The initial slope of this ternary complex growth curve yields a value for k_c of about 1×10^{-8} s^{-1} at either pH and gives a good agreement with the value estimated by our model. Moreover, the fraction of slow to fast components plateaus at 0.33 and 0.22 at longer times for pH values of 7.5 and 5.5, respectively. These values follow from our estimated values for P_T/R_T shown in Figure 3, combined with the ternary complex affinity and receptor/ligand affinity.

TABLE I. EGF Receptor Binding Parameters

$k_f(M^{-1}s^{-1})^b \times 10^{-6}$	$k_r(s^{-1})^c \times 10^2$	$K_{d1}(M) \times 10^8$	$k_c(s^{-1})^d \times 10^8$	$k_u(s^{-1})^c \times 10^5$	$K_{d2} \times 10^{-3}$
pH 7 5					
1 9 ± 6[a]	1.8 ± .9	1 ± .4	1 1 ± 9	2.9 ± 2	2 6 ± 1
pH 5					
1 8 ± 6	4 ± 1.5	2 ± 1.6	.9 ± .7	2 7 ± 2	3 ± 1.2

[a]Errors are standard deviations averaged over three experiments
[b]Derived from initial slopes of kinetic binding data
[c]Estimated from dissociation kinetic data
[d]Estimated from the initial slope in Fig 6

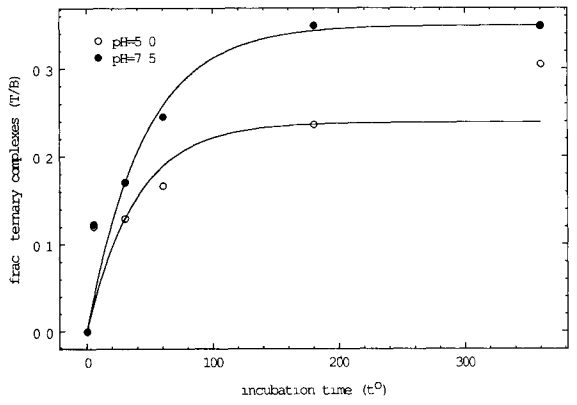


Fig. 6. Fractional change in ordinate intercept of figure 5 vs incubation time The ordinate intercept of each plot representing the fraction of the slow component dissociated (dashed lines, Fig 5) per incubation time is plotted here The ordinate is the ratio of ternary complexes (T or T_s as defined by equation 1) to total complexes (B for bound = $C_s + T_s$ as defined by equation 1) The initial slope gives a rate constant value of $k_c = 1\ 2 \times 10^{8}\ s^{-1}$ Data are shown for pH 7 5 and pH 5

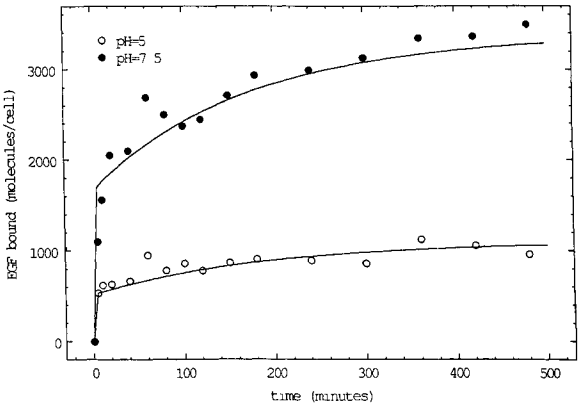


Fig. 7. Ternary complex model fits to EGF binding kinetics at pH 7 5 and 5 Ternary complex model fits to EGF binding kinetic data are shown for pH 7 5 and 5

The fraction of ternary complex formed per unit of total complex is greater at pH 7.5 or 8 than at pH 5, indicating that ternary complex formation is more than a linear function of the amount of binary complex formed. The fact that the fraction of ternary complex formed as a function of total complex formation was higher in the case where total binding was higher provides an indication that ternary complex formation is not limited by the concentration of the membrane "interaction molecule." Interestingly, it appears from Figure 6 that ternary complex is formed in abundance at either pH 5 or 7.5, although high affinity binding is observed only at higher pH values. The reason(s) for this is unclear, but probably lies in the inability of ordinate-intercept data (Fig. 5) to quantitate ternary complex formation. At best, these data can only qualitatively suggest a pH-dependent decrease in the fraction of ternary complexes relative to EGF-receptor bound species. That Scatchard plots at lower pH values do not show any significant high affinity species may simply be the result of decreased numbers of ternary complex molecules and the insensitivity in the analytical technique used for their detection. Identity of the interaction molecule itself remains unknown, although it could be a coated pit protein [Mayo et al., 1989]. These data cannot exclude the possibility that it may even be occupied EGF receptor dimer (Yarden and Schlessinger, 1987).

Ternary Complex Model Fits to Kinetic Binding Data

Figure 7 shows pH 7.5 and pH 5 kinetic binding data which have been fit with the ternary complex model [Mayo et al., 1989]. The only model parameters which had to be varied significantly in order to obtain a satisfactory fit to both curves were k_r, the EGF-receptor dissociation rate constant, and P_{tot}, the apparent number of accessory molecules.

Sensitivity analysis on these binding data, which shows the effects of perturbing the rate constants, k_r and k_u, and also P_{tot} and R_{tot}, by 10–100%, indicates that k_r is most sensitive to change. This supports results discussed above that simple dissociation of the receptor/ligand complex plays the major role in the pH effect on receptor binding and ternary complex formation.

DISCUSSION

Consideration of the pH dependence of extracellular EGF-receptor binding can aid in our understanding of intracellular EGF-receptor trafficking. The pH of endocytotic vesicles containing EGF has not been measured directly, but in fibroblasts, EGF enters the same endocytotic vesicles as α_2-macroglobulin [Maxfield et al., 1978] which is known to have a pH of about 5 [Tycko and Maxfield, 1982]. Endocytotic internalization, therefore, probably introduces EGF-receptor complexes to pH values around 5 where dissociation is known to occur [Haigler et al., 1980; Maxfield, 1982]. Upon internalization, EGF-receptor dissociation is still considered by many, however, to be complete or near complete. These present binding data clearly show this not to be the case. In fact, on reducing the pH from 8 to pH 5, the amount of bound EGF receptor is only decreased by about a factor of four. This result is consistent with recent evidence showing that mis2 intracellular receptors remain bound by EGF [Sorkin et al., 1991]. The combination of the fairly weak pH dependence of the EGF-receptor complex affinity, with the large effective ligand concentrations present within the endosome, should indeed favor continued occupancy [Linderman and Lauffenburger, 1988].

Of possible relevance to the understanding of cell surface and cytoplasmic dynamic processes is the pH dependence of individual steps in the ternary complex model [Mayo et al., 1989]. These results indicate that the initial EGF-receptor binding process from equation 1 (i.e., K_{d1}):

$$L_o + R_s \underset{k_r}{\overset{k_f}{\rightleftarrows}} C_s$$

apparently contributes most to the observed pH dependence of EGF receptor binding. In fact, model parameterization in terms of k_r is the most sensitive variable in fitting the experimental data. While other terms vary somewhat, k_r is increased 3–5 times on going from pH 8 to pH 5. This increase in the dissociation rate at lower pH relates to weaker receptor binding. In turn, this suggests that either the EGF receptor, EGF itself, and/or the EGF-receptor membrane environment undergoes some pH-induced conformational change(s) and/or that pH-induced electrostatic differences in surface charged groups are responsible for decreased binding. Mass action then dictates that this decrease in the bound EGF-receptor population (i.e., C_s) must be reflected in the ternary complex population which is clearly attenuated below pH 6 as evidenced in the equilibrium Scatchard plots by a reduction in the high affinity fraction [Mayo et al., 1989].

Dipaola and Maxfield [1984] reported that lowering the extracellular pH from 7.0 to 5.6 makes the EGF receptor more resistant to papain proteolysis and decreases the amount of lactoperoxidase-catalyzed receptor iodination. Based primarily on these results, they concluded that EGF receptors become less exposed to the external aqueous environment at lower pH. This hypothesis is in general agreement with our results which indicate that the EGF-receptor dissociation rate is increased, and therefore receptor binding affinity is decreased, by lowering the pH from pH 8 to pH 5. In biological terms, a factor of four, while significant, is not relatively large. Slight changes in and/or around the EGF-receptor environment could possibly account for such differences in receptor binding levels. At the same time, we have found a pH effect on the apparent number of accessory molecules which are available to participate in ternary complex formation (see Fig. 3). Though computer fits to our binding data were much less sensitive to P_{tot} values than to k_r values, a decrease in this parameter as pH is lowered may be at least partially responsible for decreased binding. If multiple classes of accessory molecules exist, it is possible that receptors are prevented from interacting with some, but not all, classes by the pH effect.

Wiley and Cunningham [1987] have observed that the EGF receptor internalization rate is also reduced by lowering the extracellular pH. Internalization follows receptor binding and ternary complex formation. A reduction in the T_s population caused by a reduction in both ligand/receptor affinity and apparent number of accessory molecules would explain such a reduction

in the receptor internalization rate. Recently, Lund et al. [1990] showed that internalization of EGF receptors occurs through at least two distinct pathways: a low capacity pathway that has a relatively high affinity for occupied receptors, and a low affinity pathway that has a much higher capacity. The high affinity pathway was observed in all cells having receptors with intrinsic tyrosine kinase activity. Mutant EGF receptors lacking kinase activity could not utilize the high affinity pathway and were internalized only through the low affinity one. Kinase activation has also been shown to produce the observed pH-dependent concave-upward Scatchard binding curves [Wiley et al., 1991]. It may be that pH can act to affect kinase activity by modulating EGF receptor binding. This merits further investigation.

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