

High-Affinity Epidermal Growth Factor Binding Is Specifically Reduced by a Monoclonal Antibody, and Appears Necessary for Early Responses

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Abstract. We have tested the effects of an mAb directed against the protein core of the extracellular domain of the human EGF receptor (mAb108), on the binding of EGF, and on the early responses of cells to EGF presentation. We used NIH 3T3 cells devoid of murine EGF receptor, transfected with a cDNA encoding the full-length human EGF receptor gene, and fully responsive to EGF. The binding to saturation of mAb108 to the surface of these cells at 4°C and at other temperatures specifically reduced high-affinity binding of EGF, but did not change the dissociation constant or the estimated number of binding sites for low-affinity binding of EGF. The kinetics of EGF binding to the transfected cells were measured to determine the effects of the mAb on the initial rate of EGF binding at 37°C. Interestingly, high-affinity EGF receptor bound EGF with an intrinsic on-rate constant 40-fold higher ($9.8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) than did low-affinity

receptor ($2.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$), whereas the off-rate constants, measured at 4°C were similar. Cells treated with the mAb or with phorbol myristate acetate displayed single on-rate constants similar to that for the low-affinity receptors.

At low doses of EGF ranging from 0.4 to 1.2 nM, pretreatment of cells with mAb108 inhibited by 50–100% all of the early responses tested, including stimulation of tyrosine-specific phosphorylation of the EGF receptor, turnover of phosphatidyl inositol, elevation of cytoplasmic pH, and release of Ca^{2+} from intracellular stores. At saturating doses of EGF (20 nM) the inhibition of these early responses by prebinding of mAb108 was overcome. On the basis of these results, we propose that the high-affinity EGF receptors are necessary for EGF receptor signal transduction.

EPIDERMAL growth factor (EGF) is a polypeptide hormone that is mitogenic for a variety of cell types, including fibroblasts and epithelial cells (reviewed in Carpenter and Cohen, 1979, and Schlessinger et al., 1983). EGF exerts its effects through the EGF receptor (EGF-R),¹ a glycoprotein that resides in the plasma membrane, binds and internalizes EGF, and contains within its cytoplasmic domain a tyrosine-specific protein kinase activity. The kinase domain is homologous to a family of membrane-bound tyrosine kinase enzymes that are involved in normal growth control or have been implicated in oncogenic growth stimulation (Downward et al., 1984, Hunter and Cooper, 1985, Yarden and Ullrich, 1988). The binding of EGF to the EGF-R stimulates a series of rapid responses, including phosphorylation of tyrosine residues within the EGF-R itself and within many

other cellular proteins, hydrolysis of phosphatidyl inositol, release of Ca^{2+} from intracellular stores and in some cell types the influx of extracellular Ca^{2+} , elevation of cytoplasmic pH, and morphological changes. After 10–12 h in the continuous presence of EGF quiescent target cells are committed to synthesize DNA and to divide. The kinase activity appears a necessary part of the signal transduction process, because its inactivation by site directed mutagenesis results in an EGF-R that is normal in EGF binding and internalization, but that is completely dysfunctional in signal transduction and normal receptor routing (Livneh et al., 1986; Chen et al., 1987; Honegger et al., 1987a,b; Moolenaar et al., 1988). The mechanism whereby EGF binding activates the EGF-R tyrosine kinase and elicits early and late responses remains unresolved.

One question relating to EGF-induced EGF-R activation is the mechanism and role of high- and low-affinity EGF binding (discussed in Carpenter, 1987, and Schlessinger, 1988). The differential binding characteristic of EGF is based on a curvilinear Scatchard plot (Scatchard, 1949) for

1. *Abbreviations used in this paper:* BCECF, bis(carboxyethyl)carboxy-fluorescein; EGF-R, epidermal growth factor-receptor; HNTG solution, 20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol; PMA, phorbol myristate acetate.

¹²⁵I-EGF binding. This is suggestive of multiple populations of receptor, with different affinities for EGF. Whether EGF-R affinity is related to functionality (with respect to kinase activity or signal transduction) remains an open question. In this study, we investigate the action of two mAbs directed against the extracellular domain of the EGF-R and use them to probe the nature of EGF-R activation. We show that one antibody, mAb96, is an antagonist, that is, it competes with EGF for binding to the EGF-R, fails to stimulate any cell responses by itself, and blocks EGF-induced cell responses (presumably by interfering with EGF binding). The other, mAb108, does not compete with EGF for binding to the EGF-R. However, mAb108 has been shown to slow the growth of two human tumor cell lines in culture and in nude mice (Aboud-Pirak et al., 1988, 1989). We demonstrate that although mAb108 does not affect the binding of EGF at saturation, the antibody specifically inhibits high-affinity binding of EGF. Along with this reduction in high-affinity binding, we show that mAb108 inhibits several early responses to low, physiological doses but not to high doses of EGF. We suggest that high affinity receptors are necessary for EGF-R signal transduction.

The early responses were measured rapidly at physiological temperatures, while the Scatchard analyses were measured over a much longer time course at 4°C. Further, if the on-rates of EGF binding are the same for high and low affinity binding, the amount of EGF bound quickly would be expected to be little affected by mAb108, as the low-affinity sites so much outnumber the high-affinity sites. We therefore will report on the kinetics of EGF binding as probed at physiological temperature, and as affected by mAb108 and by phorbol ester. We show that it is an elevation in on-rate of EGF binding that is responsible for high-affinity binding rather than a reduction in off-rate, and that mAb108 and the other treatments specifically reduced the high affinity on-rate, resulting in a twofold reduction in the amount of EGF bound quickly at low EGF concentrations at 37°C. Thus high-affinity EGF binding can be demonstrated with kinetics measurements and is inhibited by mAb108 at 37°C, directly correlating with the inhibition by mAb108 of high affinity equilibrium binding and of early cell responses to low doses of EGF.

Materials and Methods

Cell Culture

Mouse myeloma and hybridoma cell lines were grown at 37°C in DME containing 1 mM sodium pyruvate, 2 mM glutamine, and 10% FCS. The other cell lines (HER14, CH71, A431, Swiss 3T3) were grown in DME containing 10% FCS. HER14 cells (Honegger et al., 1987a) are NIH 3T3 cells (clone 2.2 lacking endogenous EGF-R) transfected with a plasmid bearing the human EGF-R full-length cDNA. The cloned transfectants express ~500,000 human EGF-Rs per cell, which appear fully functional in EGF binding, internalization, signal transduction, and stimulation of mitogenesis.

Preparation of mAbs Directed against the Extracellular Domain of the EGF-R

We have produced mAbs directed against the extracellular domain of the human EGF-R by immunizing female Balb/c mice with CH71 cells (Livneh et al., 1986). These cells are CHO cells that were transfected with a plasmid bearing a cDNA construct of the human EGF receptor truncated in the intracellular domain. They express a large number of EGF-R at their surface (>10⁶/cell) as do A431 cells, but their EGF receptor molecules do not bear

the highly antigenic blood group A-related carbohydrates of A431 cell membrane proteins. Spleen cells of two immunized mice were fused with NSO-1 myeloma cells using a modified technique of Kohler and Milstein (1975). The hybridomas secreting mAbs against the EGF-receptor were selected for their ability to bind to either A431 or HER14 cells. NIH 3T3-2.2 cells, which express no measurable EGF-R, were used as a negative control. The selected hybridomas were cloned and then injected into mice to produce ascites fluid. After ammonium sulfate precipitation of the ascites fluid, two antibodies were purified: mAb96, (IgM) by gel filtration on Sephacryl S300 (Pharmacia Fine Chemicals, Piscataway, NJ) column, and mAb108 (IgG_{2a}) by affinity on a protein A-Sepharose column.

Binding Experiments

HER14 cells were grown in DME with 10% heat-inactivated FCS (Gibco Laboratories) to confluence on 24-well plates (Costar Corp., Cambridge, MA) coated with human fibronectin. Cells were washed twice with binding buffer (DME with 0.2% BSA and 20 mM Hepes, pH 7.4), and incubated in 0.5 ml binding buffer with radioiodinated EGF (chloramine T method) or with radioiodinated mAb108 (Iodogen method) added at the desired concentrations at 4°C for 90 min. For both ¹²⁵I-EGF and ¹²⁵I-mAb108, rates of binding were tested, and at all concentrations used for our Scatchard curves binding had reached equilibrium by 90 min (data not shown). After incubations, cells were washed three times with binding buffer and then lysed and the protein was dissolved with 0.5 ml of 0.2 M NaOH. This was collected and counted in a gamma counter. Counts for each sample were automatically recorded and stored on a microVAX computer, and data were fitted with a nonlinear least squares algorithm for the Michaelis-Menten binding equation assuming one or two binding populations, and plotted in Scatchard format by the computer.

[³⁵S]Cysteine Labeling of Cells and Immunoprecipitation of EGF-R

Cells at 80% confluence in 10-cm tissue-culture dishes were washed with cysteine-free DME and grown overnight in cysteine-free DME containing 10% FCS and 50 pCi/ml of [³⁵S]cysteine (New England Nuclear, Boston, MA). Cells expressing the human EGF-R (HER14), chicken EGF-R (CK109) (Lax et al., 1988) and mouse EGF-R (Swiss 3T3) were used. The cells were washed three times with PBS and scraped into 0.5 ml of lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF) and incubated for 15 min on ice. The lysates were spun for 15 min in an Eppendorf centrifuge at 4°C. 3 mg of protein A-Sepharose per sample were swollen and washed with 20 mM Hepes, pH 7.5, and then mixed for 30 min at room temperature with goat anti-mouse antibody. After washing three times with 20 mM Hepes, pH 7.5, the protein A-Sepharose-goat anti-mouse antibody complex was further incubated with the different primary antibodies for another 30 min at room temperature and washed three times with HNTG buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol). The protein A-Sepharose/antibody complexes were then incubated with the respective labeled cell lysates for 60 min in HNTG at 4°C, washed twice with 50 mM Hepes pH 8.0, 0.2% Triton X-100, 500 mM NaCl, and 5 mM EGTA, twice with 50 mM Hepes, pH 8.0, 0.1% Triton X-100, 0.1% SDS, 150 mM NaCl, 5 mM EGTA and twice with 10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100. Laemmli's sample buffer was added to the washed immunoprecipitates which were then boiled for 4 min and separated on an SDS-polyacrylamide gel (7%). Autoradiograms of the dried gels were made on Kodak X-Omat paper (Eastman Kodak Co., Rochester, NY).

Phosphorylation of EGF-R in Living Cells

HER14 cells were grown to confluence in 35-mm culture dishes coated with fibronectin. Cells were washed once with binding buffer and incubated at 4°C for 90 min with or without mAb solutions in binding buffer. The plates were then placed in a water bath at 37°C and different concentrations of EGF were added to the cells for 2 min. The buffer was removed and cells were scraped off the plates with 0.5 ml of Laemmli's sample buffer, boiled for 5 min, and sonicated for 10 s. Aliquots of each sample were run on two different SDS-polyacrylamide gels (7%) and each gel was transferred to nitrocellulose paper. One paper was immunoblotted with RK2 (Kris et al., 1985), an anti-EGF-R antibody (Fig. 5 C) and the other with an anti-phosphotyrosine-specific polyclonal rabbit antibody (Fig. 5, A and B). Blots were then labeled with radioiodinated Protein A (New England Nuclear).

Autoradiograms of the nitrocellulose papers were made on Kodak X-Omat paper.

Cross-Inhibition of Ligand Binding

HER14 cells at confluence in 24-well plates were washed with binding buffer and were incubated for 90 min at 4°C with saturating concentrations of the mAbs or EGF. At that time either radioiodinated mAb96, or mAb108, or EGF was added at saturating concentrations (in the continued presence of unlabeled ligand), and the cells were incubated for another 90 min at 4°C and then washed three times with cold PBS. The radioactivity associated with the cells was counted in a gamma counter.

Intracellular Free Calcium Measurements, Reporting Calcium Released from Intracellular Stores

Intracellular free Ca²⁺ concentrations were measured by fura-2 fluorescence in a spectrofluorometer (Spex Industries, Edison, NJ) on line with a microVAX computer for data storage, fitting, averaging, and presentation.

Cells trypsinized and plated onto 11 × 22 mm glass coverslips (Thomas Scientific, Philadelphia, PA) 2 or 3 d before experiments were grown to near confluence in complete medium (DME without phenol red, with 10% FCS in a 7% CO₂ incubator). Slips were pretreated with human fibronectin (Rorer Biotechnologies, Inc., King of Prussia, PA) to improve adhesion. Cells were serum deprived for 24 h before experiments although starving for 6 h was found to be sufficient for maximal response to PDGF presentation (data not shown).

Cells were washed once with incubation buffer (20 mM Hepes, pH 7.2, 140 mM NaCl, 1 mM CaCl₂, 5 mM KCl, 1 mM MgCl, and 10 mM glucose) and loaded with fura-2-AM (Molecular Probes, Inc., Eugene, OR). 1 μl of a 2.5 mM stock solution of fura-2-AM in dry DMSO (fresh daily) was added to 1 ml incubation buffer containing 1% BSA 0.04% pluronic 20 detergent. This 2.5-μM solution of FURA-2-AM was vortexed for 1 min and used immediately. Cells were incubated with this loading buffer for 30 min at 25°C in the dark. After loading, cells were washed four times over a 10-min period with incubation buffer warmed to 37°C. 1 min before being recorded, cells were washed once and placed in the fluorometer cuvette as described above in incubation buffer without CaCl₂, but with 2 mM EGTA instead. Hence all measurements were performed without external Ca²⁺, but before intracellular stores of Ca²⁺ were depleted.

An 11 × 22 mm coverslip with loaded cells was placed standing on end into a 10 × 10 mm wide by 30 mm high quartz cuvette, with the slip seated snugly in the front corner of the cuvette along one vertical edge and against the center of the opposite wall of the cuvette along the other vertical edge. Glass clips were pulled from 25-μl micropipettes (Drummond Scientific, Broomall, PA) and were used to hold the slip firmly in place. With this geometry, approximately two-thirds of the surface area of the coverslip was used. The cells were excited by the incident beam 15°C from normal, and fluorescence emitted at an angle of 45° from normal was collected. The fluorescence was excited at 340, 355, and 370 nm, and emission was recorded at 510 nm with 3-nm band widths. A long-pass filter, OG455 (Ditric Optics, Hudson, MA), was added to the emission path to cut down on the contributions of scattered and reflected low wavelength light, not sufficiently reduced by the monochromators.

Cellular autofluorescence for excitation at each wavelength (measured on unloaded cells) was ~10% of the measured values, and was subtracted from the measurements. Calcium concentration was estimated by comparison of the ratio of fluorescence with excitation at 355 nm, which was found to be the apparent isofluorescence point for the fluorometer and bandwidths used. The 380/355 and 340/355 ratios were compared with those ratios obtained for standard CaCl₂/EGTA solutions and 1 μM fura-2 salt measured with our equipment according to the method of Grynkiewicz et al. (1985). The estimates of the Ca²⁺ concentration from each of two ratios provided an advantage in two ways: At low Ca²⁺, the fluorescence at 340 nm excitation is low and therefore less accurate. The same is true for high Ca²⁺ and 380 nm excitation. The accuracy can thus be improved at high and low Ca²⁺ concentrations by using the average of the Ca²⁺ estimates from each ratio weighted by the relative intensity at 380 and 340, respectively. In addition, by comparing the Ca²⁺ estimates from the ratios of 380/355 and 340/355 fluorescence, data could be screened. Data that yielded inconsistent estimates from the two ratios (due presumably to poor estimates of the background fluorescence or poor loading) were not used. After 30 min of loading with FURA2, cells to be pretreated were cooled to 4°C and 30 nM mAb108 was added for 10 min. These cells were then washed with the same procedure above, but with mAb108 added at the same concentration.

Intracellular pH Measurements

The measurement of pH by the use of BCECF fluorescence on cells attached to coverslips within a cuvette was as previously described (Moolenaar et al., 1988).

Turnover of Phosphatidyl Inositol

Total [³H]inositol phosphates were measured as described (Tilley et al., 1988). Briefly, cells plated in a 35-mm dish were labeled for 48 h with 4 μCi [³H]inositol, and stimulated with different levels of EGF in the presence of 10 mM LiCl for 30 min, and the reaction stopped by addition of ice-cold TCA (10% wt/vol). Soluble material was washed with diethylether and neutralized extracts were processed for analysis of total [³H]inositol phosphates by anion-exchange chromatography (Tilley et al., 1988).

Cell Treatments for Kinetics Studies

Cells at confluence on 24-well plates were washed once in DME without serum and then incubated in this medium plus 100 nM PMA (diluted from a 100 μM stock solution in 10% DMSO) at 37°C for 45 min. Equilibrium or kinetics experiments were then performed immediately in the absence of PMA. Cells were treated with mAb108 as follows. Immediately before experiments, cells were washed in DME without serum at 4°C and incubated in this medium plus 30 nM mAb108 at 4°C for 30 min. Experiments were then performed in the continued presence of this concentration of mAb108.

Dissociation Experiments

Cells were allowed to bind radioiodinated EGF for 90 min as for equilibrium experiments. 5.0 ng/ml (= 0.83 nM) radioiodinated EGF was used for all experiments. Where indicated, 120 ng/ml unlabeled EGF was added to yield a final ligand concentration of 20.6 nM. Then cells were washed three times with 1.5 ml of cold binding buffer over a 3-min period and were left in 1.5 ml of this buffer plus 50 nM unlabeled EGF. At various times cells were washed twice with cold binding buffer over 1 min, solubilized, and counted as for equilibrium experiments. Experiments were performed in duplicate and at least twice. The 3-min wash after binding was used to allow sufficient time for the dissociation of low-affinity nonspecific interactions. In experiments performed in the presence of excess unlabeled ligand washed three times in 15 s, we have seen a decay time for these nonspecific interactions of 0.2–0.3 min (unpublished results). Using the 1-min wash procedure, the contribution of these transitory nonspecific interactions was reduced. Parallel data taken in the presence of 1 μg/ml unlabeled EGF were subtracted from all data to correct for nonsaturable binding.

Association Experiments

Cells were washed twice in binding buffer at 37°C. Cells were then incubated in 1 ml of 37°C binding buffer with radioiodinated EGF in a 37°C water incubator. The 1-ml volume was added quickly to individual wells with 15 or 30 s delay between time of addition to replicate wells. At various times (ranging from 30 s to 10 min) after the binding was begun each individual well was washed twice with 1.5 ml binding buffer at 4°C and the cells were solubilized and counted as for the other experiments. As mentioned above, the 1-min wash after binding was used to decrease the contribution of low-affinity nonspecific interactions. With this 1-min wash, nonspecific binding remained at 20% of the lowest specific binding values measured. In trial experiments using only a 10-s wash period, nonspecific binding was 50% of total binding (with no difference in the shapes of specific binding curves). Because the off-rate of EGF binding ranged from 40 to 60 min of half time at 4°C, the use of a 1-min wash could have allowed no more than 2% of specifically bound ligand to have dissociated. Moreover, as the concentration of radioligand was reduced by >100-fold within 7 s (by the two 1.5-ml washes), the binding of additional ligand that occurred during the 1-min wash must have been <2% of that bound within the first (30 s) incubation time.

Calculations and Fitting Procedure

For the association experiments concentrations of radioiodinated EGF ranged from 0.11 to 27 nM. Two 125-μl aliquots of each dilution were counted and used to normalize data. Counts bound (average of two determinations) minus counts bound in the presence of 1 μg/ml unlabeled EGF were divided by counts added to calculate the fraction of the initial EGF concen-

trations bound ("counts added" disagreed by <10% from each targeted concentration). Fractional specific binding data were fit by nonlinear least squares (Bevington, 1969) to the equation in the legend to Fig. 8. This equation was integrated numerically to calculate the function value at each time and EGF concentration. Integral summation was performed at 0.1-s increments (values were changed <0.5% by integrating at 1- or 0.1-s increments). Untreated cells and cells pretreated with mAb108 were assayed on the same day from cells plated at the same time, with the same dilutions of radioligand while cells treated with PMA were assayed on a different day at a different cell density.

Results

Characterization of the mAbs

Fig. 1 demonstrates the specificity of mAb96 and mAb108 towards the human EGF-R. Both antibodies specifically precipitated the M_r -170,000 human EGF-R from HER14 cells and not the murine or chicken EGF-R from Swiss 3T3 cells expressing endogenous murine EGF-R or CER cells expressing chicken EGF-R (Lax et al., 1988, 1989), respectively. In contrast, RK2 (Kris et al., 1985), a rabbit polyclonal antibody directed against a peptide within the intracellular domain of the EGF-R precipitated the EGF-R from all three species. Both mAb96 and mAb108 precipitated a nonglycosylated M_r -130,000 protein from HER14 cells pretreated with tunicamycin to prevent glycosylation (data not shown).

The data presented in Table I show that mAb96 and mAb108 do not compete for binding to the EGF-R. Whereas mAb96 and EGF compete with each other efficiently, mAb108 and EGF do not. Hence mAb96 and EGF bind at or near the same site, whereas mAb108 binds at a distinct site. Further, mAb108 can immunoprecipitate ^{125}I -EGF if cross-linked to the receptor, demonstrating that both ligands can bind to the same receptor molecule (data not shown).

Radioiodinated mAb108 bound to the EGF receptor on the surface of HER14 cells with a dissociation constant (K_d) of

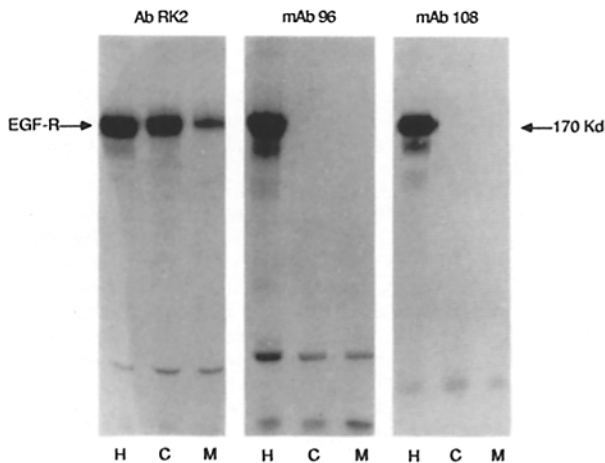


Figure 1. Specificity of Anti-EGF-R mAbs mAb96 and mAb108. Immunoprecipitates of solubilized extracts from [^{35}S]methionine-labeled cells were analyzed by SDS-polyacrylamide gels (7%). Cells expressing the human (H), chick (C), and mouse (M) EGF-R were used. Unlike Ab RK2 (directed against the intracellular kinase domain of the EGF-R), both mAb96 and mAb108 were specific for the human receptor.

Table I. Cross Inhibition of Saturable Binding

Unlabeled competing ligand	Radiolabeled ligand		
	^{125}I -EGF	^{125}I -mAb96	^{125}I -mAb108
Control	555*	1,008	482
330 nM EGF	—	107 (90%)	524 (-9%)
1 μM mAb96	0 (100%)‡	—	415 (14%)
6 μM mAb108	433 (22%)	945 (6%)	—

HER14 cells grown to confluence in 24-well plates were preincubated at 4°C for 90 min with unlabeled mAb96, mAb108, or EGF at the saturating doses listed. Then radioiodinated mAb96, or mAb108, or EGF was added to each well, and incubation continued for another 90 min at 4°C. Final concentrations of radioligand were 80 nM ^{125}I -EGF, 18 nM ^{125}I -mAb108, and 3.4 nM ^{125}I -mAb96. The radioactivity that remained cell-associated after 3 min of washing was counted in a gamma counter.

* Counts per minute $\times 10^{-3}$ cell associated, after subtraction of nonsaturable binding (counts bound in the presence of excess of the same unlabeled ligand; <20% of total binding for each radioligand. Values are mean of triplicates, SD <5% of each mean).

‡ Percent inhibition.

2.0 nM, and an estimated number of binding sites of 4.6×10^5 (Fig. 2). This number of sites is roughly equal to the number of sites for EGF, suggesting that mAb108 binds with a 1:1 stoichiometry to all of the EGF-R on the surface of these cells. The dissociation constant for mAb96 was found to be 0.8 nM (data not shown). In the presence of EGF, the affinity of mAb108 was reduced to a K_d of 5.0 nM, whereas the number of sites was little changed (Fig. 2).

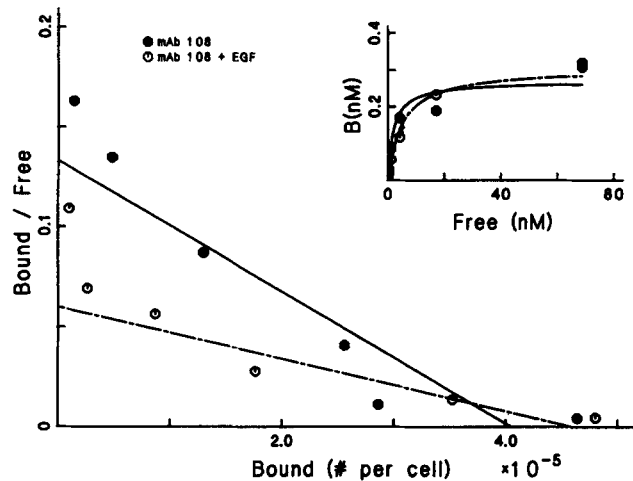


Figure 2. Binding of mAb108 to HER14 cells. The binding of radioiodinated mAb108 to HER14 cells is plotted as bound versus free (inset) and in Scatchard analysis for control cells (●) and cells preincubated and incubated in the presence of 30 nM EGF (○). Details are presented in Materials and Methods. The values of the fitted equilibrium dissociation constants were 2.0 nM without EGF and 5.1 nM in the presence of EGF. The numbers of binding sites were 4.1×10^5 and 4.6×10^5 , without and with EGF, respectively. Curves were fit for simplicity of interpretation to the binding equation assuming a single affinity, even though both Scatchard curves appear curvilinear. The nonlinearity appeared both with and without EGF, and was reproducible. The origin of this effect is not known, but may be due to a reduction in the stoichiometry of antibody binding with increasing concentration. This effect appears to have nothing to do with the dual affinities of EGF binding, as curvilinearity remains for TPA pretreated cells (data not shown).

Effect of the Antibodies on the Binding of EGF

Fig. 3 shows that in a competition binding experiment, both mAb96 and mAb108 interfere with the specific binding of radioiodinated EGF to HER 14 cells. mAb96 completely inhibited the binding of 1.7 nM ^{125}I -EGF with a concentration at half-maximal inhibition estimated to be 0.2 nM. The presence of mAb108 led to only a 50% inhibition of binding of EGF at the concentration of radioligand used, even for concentrations of antibody >20 nM. The concentration of antibody needed to produce half of this inhibition was 2.7 nM. These effects of the antibodies on the binding of EGF were identical for the inhibition of binding of radioiodinated TGF α (Fig. 3).

To further understand the inhibition of binding of EGF by the antibodies, Scatchard analyses of the binding of the hormone with and without 30 $\mu\text{g}/\text{ml}$ of either one of the two antibodies were performed (Fig. 4). As has been shown before (reviewed in Carpenter, 1987; Schlessinger, 1986) the binding of radioiodinated EGF occurred with a nonlinear Scatchard curve, which could be fit by assuming two populations of receptor with dissociation constants, $K_1 = 0.2$ nM and $K_2 = 10$ nM, at 4°C.

Fig. 4 B demonstrates that mAb96 completely inhibited the binding of EGF at all EGF concentrations. The effect of the presence of a saturating level of mAb108 on EGF binding was quite different (Fig. 4 A). There was a considerable reduction in the amount of EGF bound at low receptor occupancy, but little reduction at high receptor occupancy. For a two-population analysis, this translates to a reduction in the assessed number of high-affinity binding sites from 19,000 to 2,000, with no discernible effect on the affinity or number of the low-affinity binding sites. In four repetitions of Scatchard binding for mAb108-pretreated cells, the number of high-affinity sites as estimated by the fitting program was consistently reduced, although to a variable degree, whereas

little effect on the estimated number of low-affinity sites was seen (data not shown). On the average the number of high-affinity sites was reduced by 9-fold and the number of low-affinity sites was reduced by 1.2-fold. As shown in the figure, the effects of mAb108 pretreatment appear similar to that of pretreating cells with one of the active phorbol esters, phorbol myristate acetate (PMA). We note that mAb108 binding to HER-14 cells is unchanged after treatment with PMA (data not shown).

Effect of mAb108 Binding on Early Responses to EGF Autophosphorylation of the EGF-R

Fig. 5 displays the effects of mAb108 pretreatment on one of the earliest responses to EGF, the autophosphorylation of the EGF-R. Autophosphorylation of EGF-R was stimulated for 2 min with EGF, and was analyzed by immunoblot with anti-phosphotyrosine-specific polyclonal rabbit antibodies (Fig. 5, A and B). The M_r -170,000 band was demonstrated to be the EGF-R of unchanging quantity by immunoblotting with an anti-EGF-R antibody, RK2 (Fig. 5 C). Fig. 5 D presents these results after quantitation by counting of gel slices. mAb108 inhibited the response to the lower concentrations of EGF, 0.3 and 0.8 nM by 52 and 43%, respectively. At the larger doses, however, mAb108 had no effect. On the other hand, mAb96 blocked the response to fully saturating EGF (data not shown).

Release of Calcium from Intracellular Stores

Fig. 6 A demonstrates the effect of mAb108 on the EGF-induced change in intracellular free Ca^{2+} concentration. The dose response of EGF showed that at high EGF concentrations, the response was larger in magnitude and occurred more rapidly. Just as for autophosphorylation activity, at the lower concentrations of EGF, mAb108 pretreatment inhibited the response, whereas at the higher concentrations of

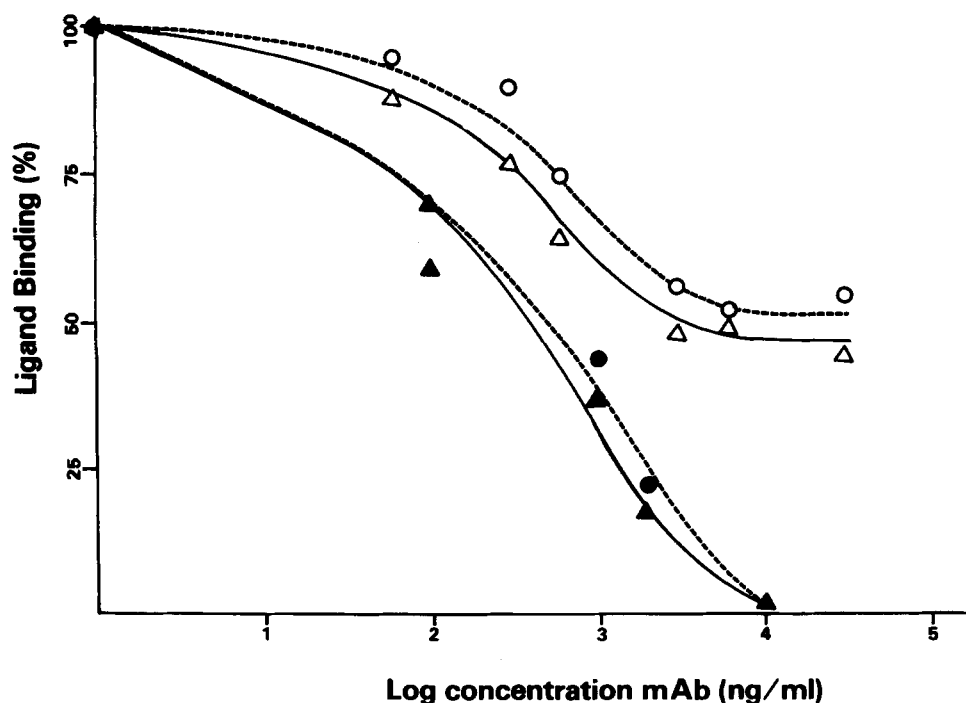


Figure 3. Inhibition of EGF and TGF binding by mAb96 and mAb108. The amount of specific binding of radioligands to HER14 cells is plotted. Cells were preincubated either with affinity-purified mAb108 (open symbols) or with mAb96 as isolated IgM (closed symbols) for 90 min at 4°C, and then 1.7 nM radioligand was allowed to bind to the cells for 90 min at 4°C. Plotted are: ^{125}I -TGF α binding to cells pretreated with mAb108 (\circ), ^{125}I -EGF binding to cells pretreated with mAb108 (Δ), ^{125}I -TGF α binding to cells pretreated with mAb96 (\bullet), ^{125}I -EGF binding to cells pretreated with mAb96 (\blacktriangle). The molar concentrations at which 50% effect occurred were 2.7 and 0.2 nM for mAb108 and mAb96, respectively.

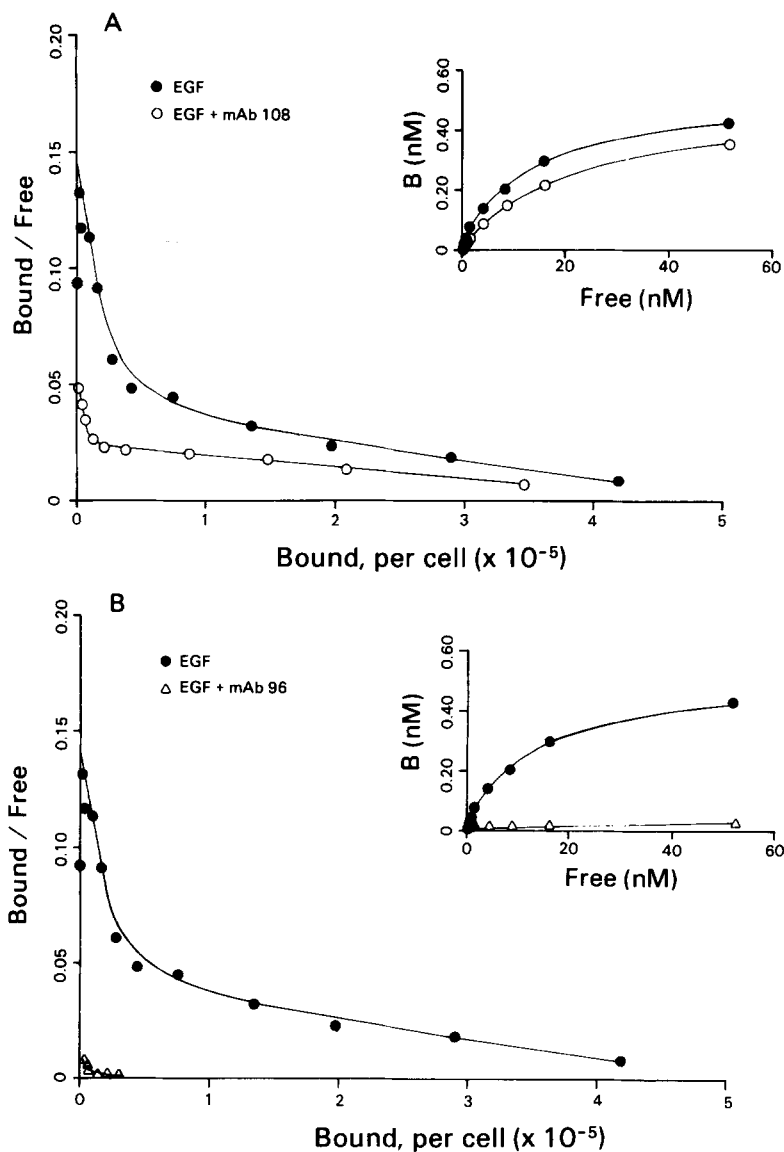


Figure 4. Effect of antibody pretreatment on EGF binding. The binding of radioiodinated EGF to HER14 cells is plotted in bound vs. free format (*insets*) and in Scatchard analysis. Plotted are the binding curves for control cells (●) and for cells pretreated and incubated either with 30 nM mAb108 (○, *A*) or with 6 nM mAb96 (△, *B*). By nonlinear least squares fitting to the binding equation (without inversion) assuming two populations of receptor, the low-affinity population bound EGF with a dissociation constant (K_d) of 14 nM, at 500,000 sites per cell. In the presence of mAb108 these values were little changed, to 22 nM at 490,000 sites per cell. The high affinity population bound EGF with K_d of 0.17 nM at 19,000 sites per cell without antibodies. In the presence of mAb108, these values were reduced to 0.04 nM and 1,900 sites per cell.

EGF, the inhibition was overcome. mAb96 blocked the response to even the highest level of EGF.

Production of Inositol Phosphates

The amount of total inositol phosphates formed after 30 min of stimulation with different concentrations of EGF is shown in Fig. 6 *B*. The dose response curve was similar to that for Ca^{2+} release and autokinase activity. At 0.8 nM EGF, the lowest dose of EGF eliciting a response, mAb108 blocked the response. At the higher doses, however, the antibody had little effect.

Elevation of Cytoplasmic pH

Fig. 6 *C* shows the dose-response data for the amount of the change in cytoplasmic pH stimulated by EGF and the effects of mAb108 pretreatment. The dose response is very similar to that for all the other early responses tested. At the low doses of 0.8 and 1.7 nM EGF, the responses were inhibited by 55 and 45%, respectively. At the saturating dose of 17 nM EGF, however, no inhibition was seen.

The effects of mAb108 were hence the same for all of the early responses to EGF tested. At the lowest concentrations of EGF, at those concentrations approaching the level of EGF *in vivo*, mAb108 exerted an inhibitory effect, ranging from 50 to 100%. For all early responses, inhibition was overcome with higher, saturating levels of EGF.

Kinetics of EGF Binding

To determine the effects of mAb108 on the amount of EGF bound quickly at 37°C and to more directly relate the short-term effects with the effects on EGF binding, the kinetics of EGF binding were measured. The dissociation rates (Fig. 7) had to be measured at 4°C (to prevent internalization), whereas the association rates were measured directly at 37°C.

Surprisingly, the rate of dissociation was little affected by the presence of mAb108, by PMA pretreatment or by changes in receptor occupancy. The pretreatments and high occupancy would be expected to report dissociation from low-affinity sites, and low receptor occupancy that from high- and low-affinity sites in roughly equal proportion (according to the equilibrium binding data, Defize, 1989).

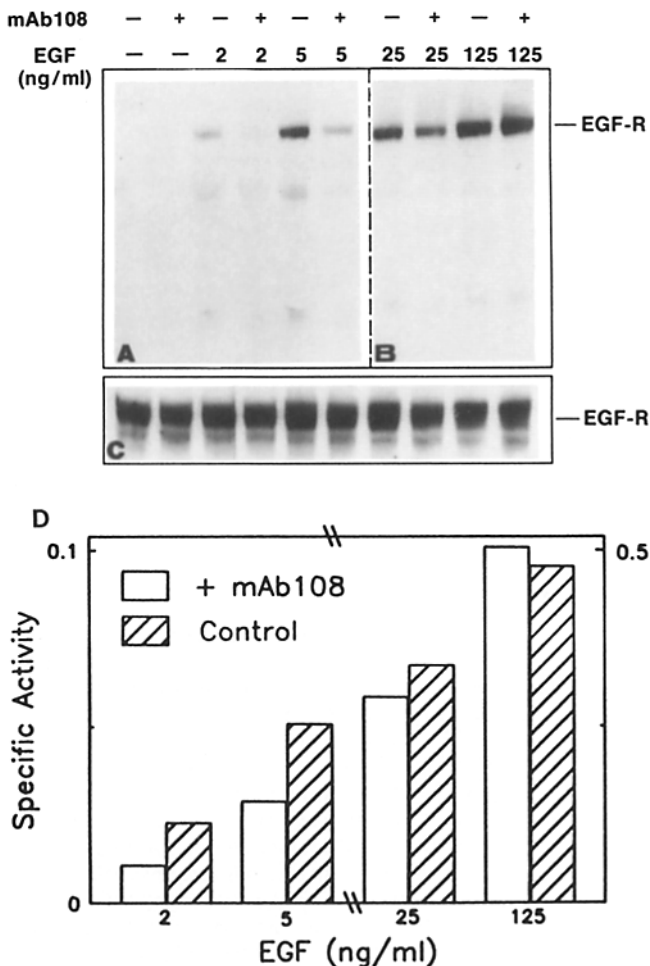


Figure 5. Autophosphorylation in living cells. HER14 cells were either pretreated with 30 nM mAb108 for 30 min at 4°C (+ mAb108, open bars) or pretreated with buffer alone (– mAb108, cross-hatched bars), and then stimulated for 2 min with various levels of EGF. SDS-polyacrylamide-separated proteins transferred to nitrocellulose paper were immunoblotted with anti-phosphotyrosine antibodies (A and B) or with anti-EGF-R (RK2) antibodies (C). For improved comparisons, the phosphotyrosine blot was exposed twice, the left side (A) for 6 h and the right side (B) for 2 h. The specific phosphotyrosine activity for each treatment is plotted in D. Bands cut from the blots were counted and the specific activity was calculated for each band as the ratio of the counts by phosphorylation blot divided by the counts for the same band by anti-EGF-R blot after subtraction of nonstimulated counts. The percents of inhibition of EGF-stimulated phosphokinase activity by mAb108 analyzed in this way were 52, 43, 13, and –5% for stimulation with 2, 5, 25, and 125 ng/ml (0.33, 0.83, 4.1, and 21 nM) EGF, respectively.

Fig. 8 displays the results of short-term association experiments measured at 37°C. Data (symbols) and fitted values (lines) were plotted as fraction of total ligand bound as a function of time. The data were fit as described in Materials and Methods to the equation given in the figure legend. The fitted values for these experiments are listed in Table II along with the apparent dissociation constants (K_d) calculated from these parameters.

For a ligand binding with a single on-rate constant, the initial slopes of the fraction of ligand bound as a function of time would be the same, that is, the initial rate of ligand binding would be proportional to ligand concentration. This was

not the case for the untreated cells (Fig. 8 A). The early data points and fitted curves displayed a higher initial rate of fractional binding at the lowest EGF concentrations. These data were fit to five parameters (refer to the equation in the figure legend), including separate terms for high- and low-affinity binding. The parameters are listed in Table II. The inclusion of the two terms for high-affinity on-rate constant and high-affinity receptor concentration decreased χ^2 by 3.6-fold, and by *F* test (Bevington, 1969), there was a probability of only 1% that the improvement of the fit was random ($P_F = 0.01$). Fitting of these data with a single on-rate but different off-rates for high- and low-affinity receptors did not significantly improve the fit ($P \cong 0.5$, data not shown). Treatment of the cells with PMA (Fig. 8 B) or with mAb108 (Fig. 8 C) reduced the binding curves to EGF binding that scaled with EGF concentration. These data were fit well with three parameters including a single on-rate. Inclusion of the two additional high-affinity terms reduced the χ^2 value by <10%, with $P_F \cong 0.5$, showing no significance to the improvement of the fit.

The fitted 37°C kinetics parameters listed in Table II agree well with the 4°C equilibrium binding results listed in the legend to Fig. 4. The dissociation constants estimated as the dividend of off- and on-rate constants agree with those directly measured at 4°C, as do the total receptor number and relative amounts of high- and low-affinity receptors. It should be pointed out that our data analysis did not include terms for internalization of EGF and receptor. Because internalization of the ligand will prevent dissociation, this omission would be expected to lead to an underestimate of the off-rate constant and apparent dissociation constants reported in Table II. However, elevation of the rate of fractional association of EGF at low concentrations was not dependent on internalization, as treatment of cells with 20 μ M phenylarsine oxide was found to inhibit internalization and slightly increase rather than decrease association by 10–20%, but not have effect on the elevation of initial rate of fractional binding at low EGF concentrations (data not shown).

Discussion

The binding of EGF to its receptor occurs with a typical nonlinear Scatchard curve, in which the amount of EGF bound at low EGF concentrations is higher than one would predict for simple binding of one ligand to one receptor of a single affinity. This characteristic holds whether the EGF receptor is expressed in its natural environment, is expressed to greatly elevated levels in naturally arising carcinoma cells, or is expressed in a foreign, cross species environment at any of a wide variety of expression levels (reviewed in Schlesinger, 1988). One simple model for nonlinear behavior of this kind is the postulation of two states of the receptor that differ in their affinity for the ligand (reviewed in Carpenter, 1987; Schlessinger, 1988). It should be pointed out, however, that no direct evidence exists to prove that there are two different populations of EGF receptor with different affinities for EGF. Other models, such as cooperative binding or the postulation of a larger number or continuous distribution of affinity states could be consistent with the observations as well. Elevation of EGF binding at low EGF concentrations is not, however, an artifact of the equilibrium binding assay, as three very different cell treatments result in linearization of the Scatchard binding curves: (a) treatment of cells with

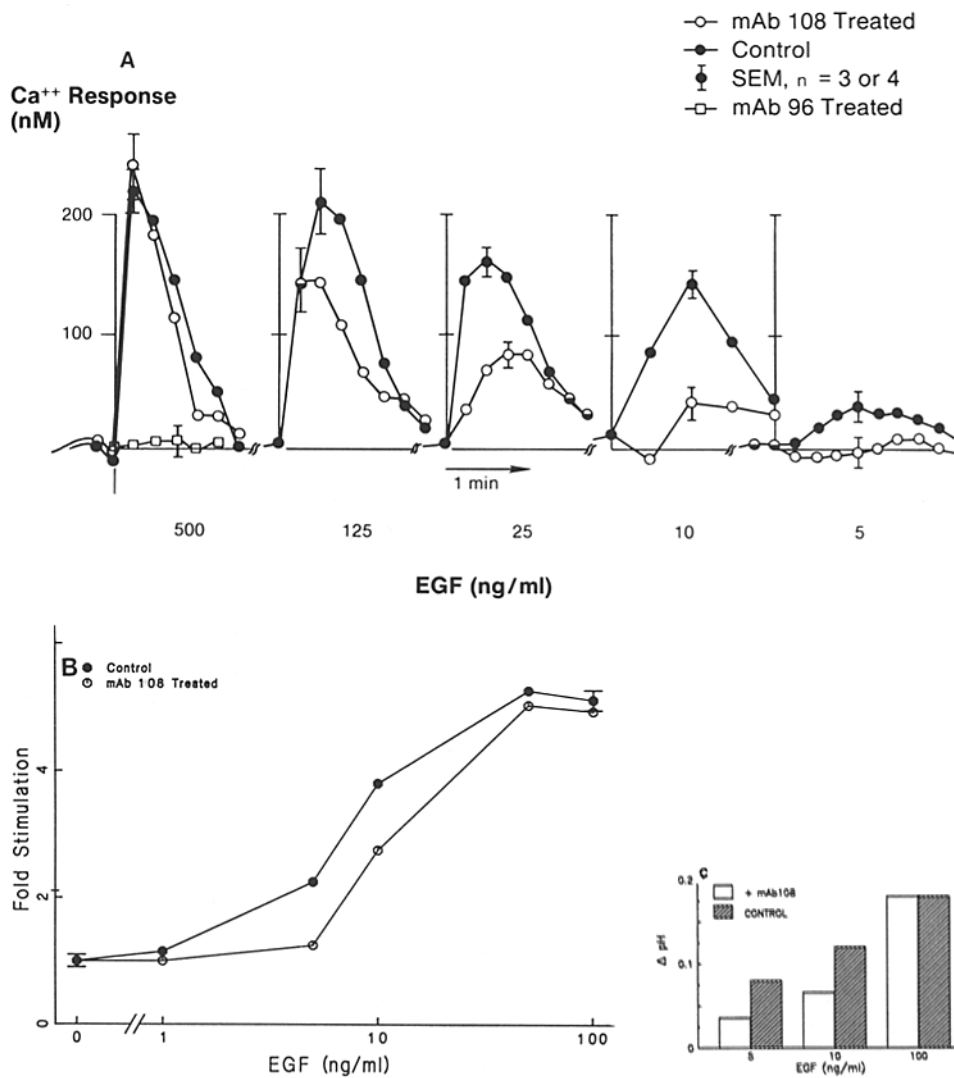


Figure 6. EGF-induced calcium mobilization, inositol phosphate production, and pH elevation. (A) The elevation of cytoplasmic Ca²⁺ levels in populations of cells in response to various levels of EGF was followed by monitoring Fura-2 fluorescence (see Materials and Methods). Cells were incubated in the absence of extracellular Ca²⁺ during the measurement. The rate and magnitude of the response increased with increasing EGF from 0.8 to 80 nM (= 500 ng/ml) EGF (●). Cells pretreated with 30 nM mAb108 (○) showed reduced and delayed responses at the lower but normal responses at the higher EGF concentrations. Cells pretreated with 1 nM mAb96 showed no response to EGF even at 80 nM EGF (□). Representative error bars (SEM, n = 3 or 4) are shown. (B) The relative amount of total [³H]inositol phosphates formed in 30 min of stimulation of cells with various levels of EGF is plotted for cells not pretreated (●) and for cells pretreated and kept in the presence of 36 nM mAb108 (○). (See Materials and Methods for details.) Representative SE bars are shown. The basal signal was 650 ± 35 cpm/10⁶ cells per 30 min. (C) The elevation of intracellular pH after 10 min of stimulation by different levels of EGF is plotted for cells pretreated for 20 min with 36 nM mAb108 (open bars) and control cells (cross-hatched bars). The ranges of the inhibition of the elevation of the pH responses were 50–60% and 40–50% for stimulation by 5 and 10 ng/ml EGF, respectively.

one of the active phorbol esters (Shoyab et al., 1979 and Fig. 4), (b) binding of EGF to EGF-R deletion mutants in which a large portion of the cytoplasmic domain had been removed (Livneh et al., 1986), and (c) as presented here the binding to saturation of an anti-EGF-R mAb. In addition, high-affinity binding can be demonstrated in the short term at physiological temperature with kinetics measurements (Fig. 8 and Table II), obviating the need for meeting the assumptions of the equilibrium experiment.

1. The Inhibition of High-Affinity Binding by mAb108

We have shown that mAb108 binds specifically to the human EGF-R without blocking EGF binding. This was unlike the results for EGF and mAb96, which compete for the same site on the EGF-R. Binding of saturating levels of mAb108 reduced the level of EGF binding at equilibrium only at low concentrations of EGF. The estimated number of high-affin-

ity receptors was reduced from 19,000 to roughly 1,900, whereas there was little effect on the number or affinity of low-affinity receptors. Conversely, the binding of EGF at saturation reduced the affinity of mAb108 two to threefold.

In addition to this analysis of the effects of mAb108 on the equilibrium binding of EGF at 4°C, we have measured the association kinetics of EGF binding at 37°C as a function of EGF concentrations. We report the same conclusions by kinetics: high-affinity EGF receptors make up 1–2% of the total, and have an affinity that is 40 times higher than that of low affinity receptors. mAb108 prebinding, similar to PMA treatment, inhibited high-affinity binding alone. Further, high-affinity receptors displayed an elevated rate of association for EGF (rather than a prolonged rate of dissociation) as compared with low-affinity receptors. mAb108 and PMA were found to inhibit the rate of EGF binding at 0.1–0.3 nM EGF by 40%, but not at all at 5–27 nM EGF (and neither agent had an effect on the rate of EGF dissociation at 4°C).

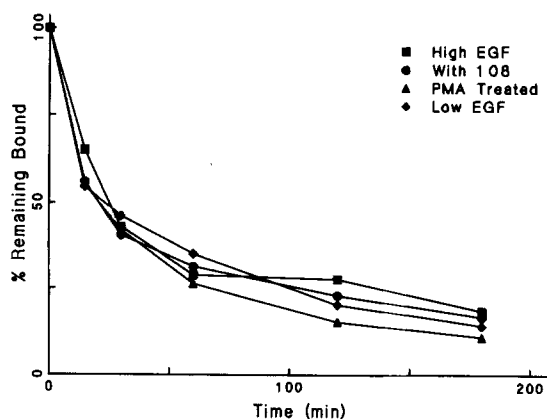


Figure 7. Rate of dissociation of EGF. After binding of ^{125}I -EGF at 4°C for 90 min, cells were washed and left in PBS with 50 nM EGF. Plotted is the amount of ^{125}I -EGF (as percent of initial binding) which remained cell associated during the wash. Cells were prebound with either 0.8 nM radioligand plus 20.2 nM unlabeled EGF (■), or with 0.8 nM radioligand (◆), or else cells were prebound with 0.8 nM radioligand after being pretreated with 100 nM PMA for 30 min at 37°C (▲), or with 30 nM mAb108 for 30 min at 4°C (●). The counts bound at zero time of wash were 1,200, 13,914, 7,502, and 7,758 for the four conditions listed, respectively. Data were fitted to exponential decay curves, yielding decay times of 20 ± 9 , 41 ± 15 , 34 ± 4 , and 35 ± 10 min, respectively, with reduced χ^2 values of 4.1, 14.2, 2.1, and 8.8, respectively.

These conclusions have been confirmed as well for A431 human carcinoma cells and for cells expressing a truncated EGF-R, which have reduced high-affinity binding (manuscript in preparation).

Hence, both by equilibrium binding at 4°C and by kinetics analysis at 37°C , mAb108 specifically inhibits high-affinity EGF binding. The molecular mechanism whereby this inhibition occurs is not clear. Because the number of binding sites for mAb108 and EGF are the same even when both are present at saturation, it is unlikely that mAb108 binds exclusively to the high-affinity receptor and directly interferes with EGF binding. If we suppose that the high- and low-affinity receptors exist in equilibrium then one possibility consistent with our data is that mAb108 binds preferentially to the EGF-R in the low affinity state, and pulls the equilibrium to favor this state. We have no data addressing this hypothesis at present.

II. The Nature of High-Affinity Binding

Contrary to the notion that receptor affinity is regulated by off-rate, we have shown that high-affinity binding is due to an elevated rate of association. Evidently the high-affinity EGF-R exists before EGF binding, and is more accessible or perhaps more electrostatically attractive to diffusing ligand. We note that our measurements of rate constants approach values measured before: the binding of EGF to placental membranes at 0.4 nM EGF has been measured at $2.6 \times 10^6 \text{ M}^{-1}\cdot\text{s}^{-1}$, and the off-rate has been measured at $6.1 \times 10^{-4}\cdot\text{s}^{-1}$, both at 23°C . (Hock and Hollenberg, 1980). By Scatchard analysis these membranes displayed a single, high-affinity with a K_d of 0.4 nM. These values are in good agreement with our values for the high affinity receptors. The existence of high- and low-affinity EGF binding has not before been demonstrated with kinetic measurements, that

is, neither the off-rate nor the on-rate of binding has been shown to be concentration dependent. An occupation-dependent rate of dissociation has been demonstrated for A431 cells, presumably due to preferential rebinding of newly dissociated hormone at moderate levels of occupancy (Wiley, 1988). However, this more common effect is most likely due to the very high local density of receptors restricted to the cell surface (Wiley, 1988; DeLisi and Wiegel, 1981), and this kind of effect cannot in itself affect the shape of a Scatchard curve (DeLisi and Wiegel, 1981). We believe the on-rate constant has not before been shown to be concentration dependent because a wide range of ligand concentrations had not been used in association-rate measurements.

Our finding that the difference between high- and low-affinity receptors is determined by the rate of association, rather than dissociation, has a precedent in growth factor-growth factor receptor interactions. High-affinity IL 2 receptors have a 1,000-fold higher affinity for IL 2 than do the low-affinity receptors, and this difference stems from a difference in the rate of ligand binding (Lowenthal and Greene, 1987). However, it should be pointed out that the molecular mechanism for this wide difference in affinity for IL 2 receptor is different from that for the EGF-R. For the former, high-affinity binding occurs to different receptor subunits, whereas for EGF-R both high- and low-affinity binding is through the same EGF-R molecule, as evidenced by the fact that cells transfected with one wild-type receptor gene display both affinities (reviewed in Schlessinger, 1986, 1988).

III. High-Affinity Receptors Are Important for Signal Transduction

Our data show that the specific inhibition of high-affinity EGF binding by mAb108 correlates with inhibition of responses to low doses of EGF for all early cellular responses tested. These results suggest that high-affinity EGF binding plays an important role in EGF signal transduction. This has been proposed before to explain why the ID_{50} for EGF-stimulated cell proliferation (measured at 46 pM for human foreskin fibroblasts, see Carpenter and Cohen, 1975) is nearly the same magnitude as the K_d for high-affinity binding, but is 2 orders of magnitude lower than the K_d for low-affinity binding. The demonstrated functionality of high affinity binding is also important physiologically, because the level of EGF is extremely low in serum, ranging from 20 to 27 pM (Hirata and Orth, 1979), and in tissues, ranging from 1 to 5 ng/g (Hirata et al., 1980). Further, two reports demonstrate that mAb108 treatment inhibits the growth of human tumor cells in culture and in nude mice (Aboud-Pirak et al., 1988, 1989). These data taken together support the notion that high-affinity EGF binding is the primary means whereby EGF stimulates cells *in vivo*.

Inhibition of high-affinity EGF binding reduces responses and cell growth, and hence high-affinity binding is used in cell stimulation, but is it necessary? Is the high-affinity EGF-R the only active moiety, or can EGF stimulate cells through interaction with the receptor in the low-affinity state? This is a difficult question, especially when the actual number of activated EGF-R molecules required is not known. As a first approach, we were interested in determining whether inhibition of short-term responses by mAb108 correlated with inhibition of EGF-R occupancy at corresponding times.

A correlation of this kind has been demonstrated between long-term EGF-R occupancy and mitogenesis (Knauer et al., 1984). To do so required measurement of the kinetics of EGF binding to these cells as affected by mAb108. As mentioned

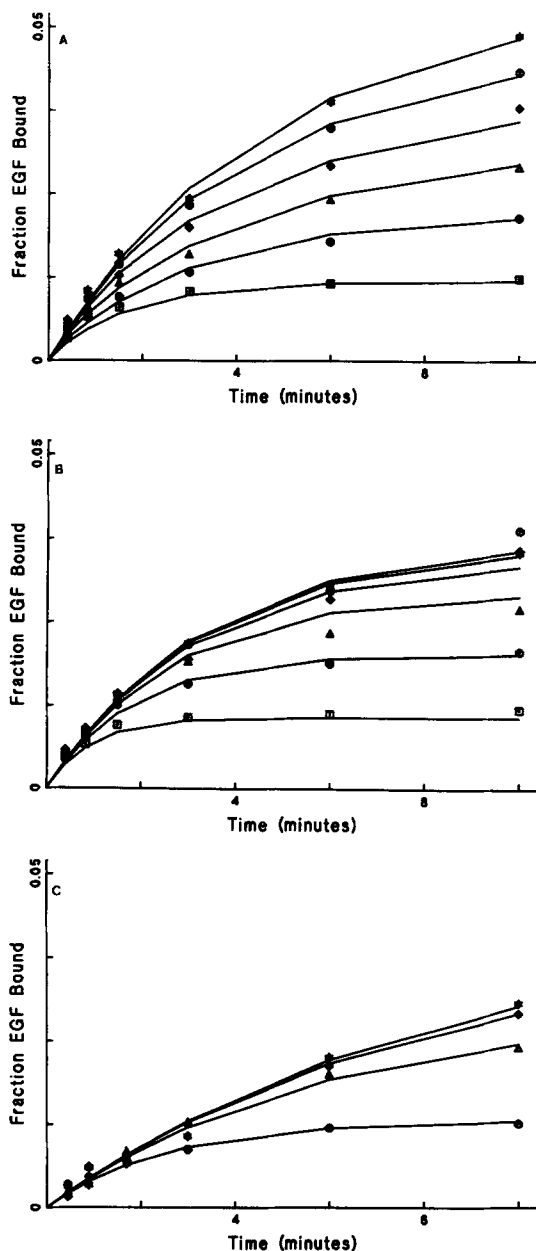


Figure 8. Rate of EGF association. The fraction of added ^{125}I -EGF specifically bound to HER14s is plotted as a function of time for control cells (A), cells prebound with mAb108 (B), and cells pretreated with PMA (C). In A and B the concentrations of EGF added were 0.11 (*), 0.33 (○), 1.0 (◆), 3.0 (Δ), 9.0 (lower ○), and 27 nM (□). In C, the concentrations were 0.1 (*), 0.5 (◆), 2.5 (Δ), and 12.5 (○) nM. Lines drawn present the fitted curves for numerical integration of the equation below giving the concentration of occupied receptor (divided by the initial EGF concentration). For control cells (A), five parameters were included in the fitting including two separate on rate constants. For treated cells (B and C), three parameters were used including only one on rate constant. The uncertainties for data points (left off for clarity) ranged for 10% of the values (for the 0.1-nM curves) to 5% for the curves with highest ligand concentrations.

above, we found that mAb108 prebinding reduced by roughly 40% the initial rate of EGF binding for low EGF concentrations (0.1–0.3 nM) but not for high concentrations (>5 nM). Hence for the early responses measured here (1–2 min except for phosphatidylinositol turnover), there is a good correlation between the amount of EGF-R occupied and the magnitude of the response, disregarding whether that receptor was initially high or low affinity. This may suggest that high-affinity receptors are important for stimulating cells at low, physiological doses of ligand, but that in their absence occupation of low-affinity receptors is sufficient. On the other hand, there may be enough high-affinity receptors left after mAb108 treatment for cell stimulation provided a higher EGF concentration is used. Alternatively, occupied low-affinity receptors may be converted to the high-affinity state before they are active.

We continue to favor the supposition that the high-affinity EGF-R is required, and restate the allosteric activation model, modified by new evidence, as a working hypothesis: the conformation of the EGF-R, which has the highest affinity for EGF and binds EGF much more rapidly, is the most active conformation in terms of kinase activity and therefore signal transduction. The binding of EGF stabilizes this species and thus elevates the concentration of the active transducer. The high-affinity active state of EGF-R appears to be stabilized by oligomerization (at least for purified receptor, Yarden and Schlessinger, 1987), by sphingolipids (Davis et al., 1988), and by EGF binding. Accordingly, EGF binding promotes receptor oligomerization (Cochet et al., 1988) and activates EGF-R, leading to a stimulation of cell growth. Finally, EGF-R in the low-affinity state, which may prefer monomeric form, appears to be stabilized by mAb108, by phosphorylation by kinase C (Davis, 1988), or by removal of a large portion of the cytoplasmic domain of the receptor (Livneh et al., 1986).

The question of the significance of EGF-R oligomerization, not directly addressed in this report, remains open. EGF binding promotes dimerization in living cells (Cochet et al., 1988), and in purified EGF-R preparations (Yarden and Schlessinger, 1987). Additionally, EGF-R dimers isolated by sucrose density gradient centrifugation bind EGF with higher affinity and demonstrate elevated kinase activity compared with monomers (Boni-Schnetzler and Pilch, 1987). However, the inhibition of high-affinity EGF binding

$$\begin{aligned} \frac{d[\text{E:R}]}{dt} &= \frac{d[\text{E:R}]_{\text{hi}}}{dt} + \frac{d[\text{E:R}]_{\text{lo}}}{dt} \\ &= k_{\text{on,hi}} [\text{EGF}] [\text{R}]_{\text{hi}} + k_{\text{on,lo}} [\text{EGF}] [\text{R}]_{\text{lo}} \\ &\quad - k_{\text{off}} ([\text{E:R}]_{\text{hi}} + [\text{E:R}]_{\text{lo}}) \end{aligned}$$

where

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

(* Fitted values.)

Table II. Fitted Kinetics Parameters

HER14	k_{on}	Receptors/Cell	k_{off}	App. K_d
	$M^{-1}s^{-1} \times 10^{-5}$		$s^{-1} \times 10^3$	nM
Control	High: 98.1 ± 2.3	7,120 (1.8%)	2.9 ± 0.2	0.30
	Low: 2.54 ± 0.38	388,000	2.9 ± 0.2	11.4
PMA-treated +mAb108	3.68 ± 0.42	354,000	1.9 ± 0.3	5.1
	4.56 ± 0.31	348,000	4.6 ± 0.3	9.6

Listed are the binding parameters fitted to the equation given in the legend to Fig. 8, along with the apparent dissociation constant, K_d , derived from dividing k_{off} by k_{on} . Uncertainties listed were as estimated by the nonlinear fitting procedure on the data shown. Receptors/cell was calculated from the fitted parameter receptor concentration, [R], by dividing by the number of cells assayed and multiplying by the volume. The reduced χ^2 (the χ^2 divided by the number of degrees of freedom) were 2.1, 3.1, and 2.3, for the control (fit to two on-rates), PMA- and mAb108-treated cells (each fit to one on-rate) respectively.

and kinase activity by PMA stimulated phosphorylation of EGF-R by kinase C, has been shown with cross-linking studies to be independent of receptor dimerization (Northwood and Davis, 1989). Perhaps nonphosphorylated EGF-R is regulated through EGF promoted oligomerization, whereas kinase C-phosphorylated EGF-R is regulated by a separate mechanism. In this regard, we have preliminary evidence to suggest that mAb108 inhibits EGF-R oligomerization as judged by cross-linking on living cells, supporting the notion that without PMA treatment, high-affinity binding and receptor oligomerization are causally related.

Received for publication 13 April 1989 and in revised form 20 October 1989.

Note Added in Proof. In a complementary study, Defize et al. (1989) recently reported that a different mAb inhibits low- but not high-affinity EGF binding, and does not inhibit early cell responses to EGF. In accord with our conclusions, those authors concluded that high-affinity EGF receptors are the active receptors in signal transduction. (Defize, L. H. K., J. Boonstra, J. Musenhelder, W. Kruijer, L. G. J. Tertoolen, B. C. Tilly, T. Hunter, P. M. P. van Bergen en Henegouwen, W. H. Moolenaar, and S. W. de Laat. 1989. Signal transduction by epidermal growth factor occurs through the subclass of high-affinity receptors. *J. Cell Biol.* 109: 2495-2507.

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