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 (mAbl08), 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 mAbl08 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 × 10^6 M^-1.s^-1) than did low-affinity receptor (2.5 × 10^5 M^-1.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 mAbl08 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 mAbl08 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), 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)carboxyfluorescein; 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 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 3T3-2.2.2 cells, which press 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 (IgG2a) 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 radiiodinated EGF (chloramine T method) or with radiiodinated 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 μCi/ml of [35S]cysteine (New England Nuclear, Boston, MA). Cells expressing the human EGF-R (HER14), chicken EGF-R (CK105) (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 at 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 protease 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 radiiodinated Protein A (New England Nuclear).
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 radiolabeled 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 Ca\(^{2+}\) concentrations were measured by fura-2 fluorescence in a spectrophotofluorometer (SpectraPhysics, 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 days before experiments were grown to near confluence in complete medium (DME without phenol red, with 10% FCS in a 7% CO\(_2\) 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 CaCl\(_2\), 5 mM KCl, 1 mM MgCl\(_2\), 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 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 CaCl\(_2\), but with 2 mM EGTA instead. Hence all measurements were performed without external Ca\(^{2+}\), but before intracellular stores of Ca\(^{2+}\) 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 contribution of these transitory nonspecific interactions was reduced. Over a 10-min period, the contribution of low-affinity nonspecific interactions. With this 1-rain wash, nonspecific interactions were 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 radiolabeled 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 radiodiagen 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 radiolabeled EGF ranged from 0.11 to 27 nM. Twenty 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 concent-

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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 2.0 nM, and an estimated number of binding sites of 4.6 \(\times 10^5\) (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 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.](image)

![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 \(\times 10^5\) and 4.6 \(\times 10^5\), 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).](image)
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 radiiodinated 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 radiiodinated TGF$\alpha$ (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 $\mu$g/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 radiiodinated 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-EGF-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 EGF the response was unchanged. We note that mAb108 binding to HER-14 cells is unchanged after treatment with PMA (data not shown).
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).
ther pretreated with 30 nM mAbl08 for 30 min at 4°C (+ mAbl08, open bars)

Figure 5. hatched bars), cut from the blots were counted and the specific activity was calcu-

the left side (A) for 6 h and the right side (B) for 2 h. The specific antibodies (A and B) or with anti-EGF-R (RK2) antibodies (C). For cellulose paper were immunobiotted with anti-phosphotyrosine an-

ments measured at 37°C. Data 25, and 125 ng/ml (0.33, 0.83, 4.1, and 21 nM) EGF, respectively.

tion of EGF-stimulated phosphokinase activity by mAbl08 analyzed after subtraction of nonstimulated counts. The percents of inhibi-

phosphotyrosine activity for each treatment is plotted in D. Bands after subtraction of nonstimulated counts. The percents of inhibi-

Figure 5. Autophosphorylation in living cells. HER14 cells were ei-

terior pretreated with 30 nM mAbl08 for 30 min at 4°C (+ mAbl08, open bars) or pretreated with buffer alone (− mAbl08, cross-
hatched bars), and then stimulated for 2 min with various levels of EGF. SDS-polyacrylamide–separated proteins transferred to nitro-
cellulose paper were immunoblotted with anti–phosphotyrosine anti-
obodies (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 calcu-
lated 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 mAbl08 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.

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 χ² 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 (P₁ = 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 = 0.5, data not shown). Treatment of the cells with PMA (Fig. 8 B) or with mAbl08 (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 χ² value by <10%, with P₁ = 0.5, showing no significance to the im-

Discussion

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 Fig. 8 displays the results of short-term association experi-

ments 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 (K₁) 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
Figure 6. EGF-induced calcium mobilization, inositol phosphate production, and pH elevation. (A) The elevation of cytoplasmic Ca$^{2+}$ levels in populations of cells in response to various levels of EGF was followed by monitoring Fura-II fluorescence (see Materials and Methods). Cells were incubated in the absence of extracellular Ca$^{2+}$ 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/10$^6$ 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 (●) 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 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.

I. 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-affinity 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).
4°C for 90 min, cells were washed and left in PBS with 50 nM EGF. These conclusions have been confirmed as well for A431 human carcinoma cells and for cells expressing a truncated EGF-R. The nature of high-affinity binding is through interaction with the receptor 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 × 10⁶ M⁻¹·s⁻¹, and the off-rate has been measured at 6.1 × 10⁻¹·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 mAbl08 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 ID₅₀ 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 $K_a$ for high-affinity binding, but is 2 orders of magnitude lower than the $K_a$ 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 mAbl08 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 mAbl08 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 mAbl08. As mentioned above, we found that mAbl08 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 mAbl08 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 mAbl08, 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

\[
\frac{d[E:R]}{dt} = \frac{d[E:R]_h}{dt} + \frac{d[E:R]_l}{dt}
\]

where

\[
[E:R]_h \text{ and } [E:R]_l = \text{concentrations of occupied high- and low-affinity receptors, respectively;}
\]

\[
*[R]_h, *[R]_l = \text{concentrations of unoccupied high and low receptors, respectively;}
\]

\[
*k_{on,h}, *k_{on,l} = \text{on-rate constants for high- and low-affinity receptors, respectively; and}
\]

\[
*k_{off} = \text{rate constant of dissociation (assumed the same for high- and low-affinity receptors, respectively.)}
\]

(* Fitted values.)

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 mAbl08 (B), and cells pretreated with PMA (C). In A and B the concentrations of EGF added were 0.11 (*), 0.33 (o), 1.0 (¢), 3.0 (zx), 9.0 (lower o), and 27 nM (D). In C, the concentrations were 0.1 (*), 0.5 (*), 2.5 (zx), and 12.5 (o) 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.
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, where-as 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, B. C. Tilly, T. Hunter, P. M. P. van Bergen en Henegouwen, W. H. Mooienaar, and S. W. de Laat. 1989. Signal transduction by epidermal growth factor receptor. Cell. 51:199-209.)

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