Structure−Function Studies of Ligand-Induced Epidermal Growth Factor Receptor Dimerization†

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Received October 15, 1997; Revised Manuscript Received December 29, 1997

ABSTRACT: We present a novel 96-well assay which we have applied to a structure−function study of epidermal growth factor receptor dimerization. The basis of the assay lies in the increased probability of EGFRs being captured as dimers by a bivalent antibody when they are immobilized in the presence of a cognate ligand. Once immobilized, the antibody acts as a tether, retaining the receptor in its dimeric state with a resultant 5−7-fold increase in binding of a radiolabeled ligand probe. When the assay was applied to members of the EGF ligand family, murine EGF, transforming growth factor alpha, and heparin-binding EGF-like growth factor were comparable with human EGF (EC_{50} = 2nM); betacellulin, which has a broader receptor specificity, was slightly less effective. In contrast, amphiregulin (AR_{1-84}), which has a truncated C-tail and lacks a conserved leucine residue, was ineffective unless used at >1 μM. We further probed the involvement of the C-tail and the conserved leucine residue in receptor dimerization by comparing the activities of two genetically modified EGFs (the chimera mEGF/TGFα_{44-50} and the EGF point mutant L47A) and a C-terminally extended form of AR (AR_{1-90}) with those of two other unrelated EGF mutants (I23T and L15A). The potency of these ligands was in the order EGF > I23T > mEGF/TGFα_{44-50} > L47A = L15A ≫ AR_{1-90} > AR_{1-84}. Although AR was much worse than predicted from its affinity, this defect could be partially rectified by co-localization of the immobilizing antibody with heparin. Thus, it seems likely that AR cannot dimerize the EGFR unless other accessory molecules are present to stabilize its functional association with the EGFR.

The epidermal growth factor receptor (EGFR)[1] is a transmembrane receptor tyrosine kinase which plays a pivotal role in the regulation of normal cell growth and differentiation. It can be activated extracellularly by one of several ligands that share a common EGF structural motif characterized by three loops imposed by intramolecular disulfide bonds (*1*). Six ligands have been well characterized to date: epidermal growth factor (EGF) (*2*), transforming growth factor alpha (TGFα) (*3*), amphiregulin (AR) (*4*), heparin-binding EGF-like growth factor (HB-EGF) (*5*), betacellulin (BTC) (*6*), and epiregulin (*7*). AR, HB-EGF, and BTC possess extended N-terminal tails that, in the case of AR and HB-EGF, include heparin binding motifs. Although it was originally believed that these ligands bind exclusively to the EGFR, BTC and HB-EGF have recently been shown to have a broader specificity binding to c-erbB4 as well as EGFR (*8*−*10*). Ligand binding to the EGFR promotes receptor dimerization which in turn results in elevation of its intrinsic tyrosine kinase activity, initiation of intracellular signal transduction, and ultimately a cellular response (*11*).

Clinical interest in the EGFR stems from the observation that it is frequently overexpressed in a variety of common carcinomas where its expression is associated with disease recurrence, reduced survival, and the presence of metastases (*12*). Given that this receptor controls many cellular processes which can be subverted by the tumor cells to promote not only their growth, but also their survival and dissemination (*13*), considerable effort has been directed toward development of novel chemotherapeutic agents capable of blocking EGFR-mediated cellular activation. Although several candidate compounds have been developed which inhibit intracellular signal transduction from the EGFR (*14*), no effective agents have been developed which are capable of blocking ligand binding or receptor dimerization. However, by working extracellularly, such reagents would offer considerable advantages; they would bypass the requirement to cross the plasma membrane in order to reach a specific intracellular compartment, they would not be exposed to drug resistance mechanisms such as the multidrug

† This work was supported by the Cancer Research Campaign, U.K. and by USDOE Contract DE-AC05-96OR22464 to Lockheed Martin Energy Research Corporation. M.B.M. was supported by NCI Postdoctoral Training Grant CA 09336, and K.N. by NSF Grant BES 9421774.

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[1] Abbreviations: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor (h or m prefix refers specifically to the human or murine sequence, respectively); TGFα, transforming growth factor α; BTC, betacellulin; HB-EGF, heparin-binding EGF-like growth factor; AR, amphiregulin; aFGF, acidic fibroblast growth factor; HEPES, *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid; ELISA; enzyme-linked immunosorbent assay; MoAb, monoclonal antibody; BSA, bovine serum albumin; TMB, 3, 3′, 5, 5′-tetramethylbenzidine dihydrochloride; HSPG, heparan sulfate proteoglycan; ATCC, American Type Culture Collection; SAXS, small-angle X-ray scattering.

S0006-2960(97)02548-8 CCC: $15.00
Published on Web 03/20/1998

resistance efflux pump, and they also offer a range of targeting options.

The mechanism of ligand-induced EGFR dimerization still remains unclear, even though a requirement for intermolecular association to achieve receptor activation was demonstrated a decade ago (*15*). From studies using the soluble extracellular domain of the EGFR (sEGFR), it has been demonstrated that dimerization requires two molecules of monomeric EGF and involves dimerization of a stable intermediate 1:1 sEGFR−EGF complex (*16*); however, the exact role of ligand in these processes is not clear. Two extreme case scenarios have been proposed: either that ligand induces a conformational change within the EGFR that exposes a site for receptor−receptor interactions or alternatively, that ligand might participate in receptor cross-linking (*16*). On thermodynamic grounds, as well as a number of other considerations, Lemmon et al. argue that ligand-mediated dimerization is more likely (*16*). If this proved to be the case, modified ligands or other small molecules might be developed which bind to monomeric EGFR but are unable to cross-link, as have already been described for the hGH receptor (*17*). Such an EGFR-directed reagent would hold significant therapeutic potential.

Although EGF has been subjected to numerous structure−activity studies (*18*), no work has specifically set out to determine whether any region of the ligand plays a major role in promoting EGFR dimerization. Given that the native EGFR ligands show considerable variation in their biological potencies (*19*) and that some mutations in EGF have been reported to have functionally different consequences [e.g., the low Vmax mutants of EGF (*20*−*23*) and the superagonistic activity of an EGF/TGFα chimera (*24*)], we set out to explore whether there might be observable differences in the abilities of these ligands to promote EGFR dimerization. Dimerization of the EGFR has been studied in a variety of experimental formats including covalent cross-linking (*25*), separation in nondenaturing gels (*26*) or sucrose gradients (*27*), fluorescence energy transfer experiments (*28*), surface plasmon resonance (*29*), and titration calorimetry and SAXS analysis (*16*). While each of these methods has its own individual merit, the majority require expensive equipment or are too cumbersome for routine use. Therefore, as a part of this study, we set out to develop and characterize a simple and inexpensive assay for routine measurement of EGFR dimerization before applying it toward the study of native and recombinant EGFR ligands. Part of this work has previously been presented in abstract form (*30*).

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Company, Poole, Dorset, U.K., unless otherwise stated. Tissue culture materials were from Life Technologies Ltd, Paisley, Renfrewshire, U.K. [^{125}I]Sodium iodide was purchased from Amersham International PLC, Little Chalfont, Buckinghamshire, U.K. ^{125}I-labeled mEGF was prepared using Iodobeads (Life Science Laboratories Ltd, Sedgewick Rd., Luton, U.K.) according to the manufacturers instructions. A431 squamous carcinoma cells were from the ATCC. EGF, TGFα, BTC, HB-EGF, AR_{1-84}, and AR_{1-90} were from R&D Systems Inc., Abingdon, Oxon, U.K.; the relative potency of these ligands was confirmed in a standard mitogenesis assay using NR6/HER fibroblasts as previously described (*31*). L47A, L15A, I23T, and mEGF/TGFα_{44-50} were prepared as previously described (*20*−*24*).

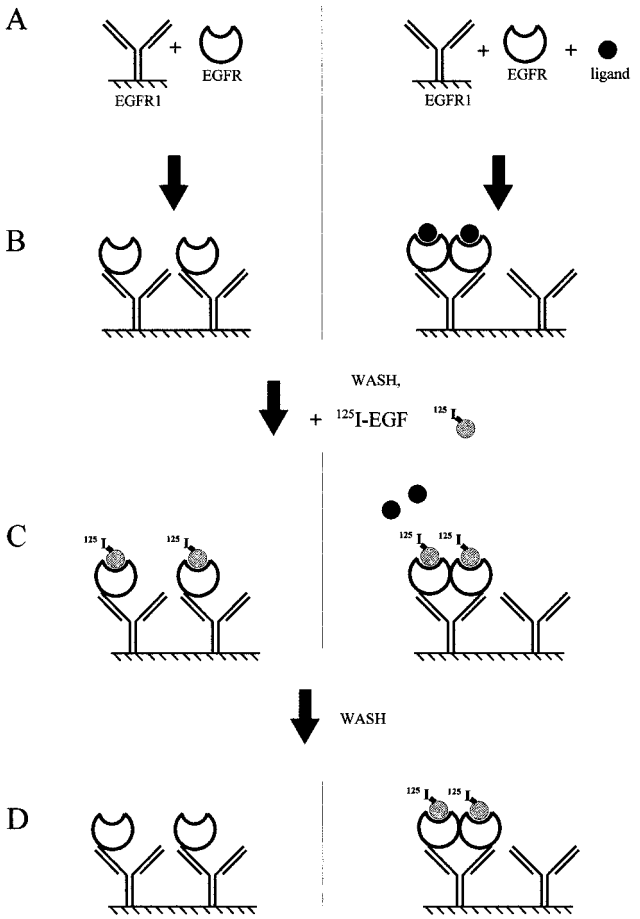

FIGURE 1: Schematic representation of the EGFR dimerization assay. (A) MoAb EGFR1 is immobilized on a 96-well tray, followed by addition of detergent-solubilized EGFR in the presence or absence of ligand. (B) In the absence of ligand, EGFR is captured predominantly in the monomeric form on separate antibodies. The ability of the ligand to promote receptor dimerization determines the proportion of dimeric receptor captured by the MoAb (see text). (C) After washing away any unbound reagents, bound receptors are probed with ^{125}I-labeled EGF. In the case of ligand-pretreated receptors, any unlabeled ligand that is still present is displaced by the excess of tracer ligand. (D) Excess tracer ligand is washed away. Low-affinity interactions such as ligand−monomer complexes are lost, leaving only ligand bound to receptor dimers.

EGFR Preparation. EGFRs were solubilized from plasma membrane vesicles prepared from A431 cells by hypotonic blebbing (*32*). The vesicles were solubilized into 20 mM HEPES containing 5% (v/v) Triton X-100 and 10% (v/v) glycerol. Samples were routinely diluted to give a working EGFR concentration containing less than 1% Triton X-100. Protein concentration was determined using the microBCA reagent with BSA as standard.

EGFR Dimerization Assay. Figure 1 shows a schematic representation of the radioligand binding assay employed. In this assay, the monoclonal anti-EGFR antibody [clone EGFR1 (*33*)] was immobilized on a 96-well tray [200 ng/well in 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.6), unless indicated otherwise in the text] for 24 h at 4 °C. When required as an Fab fragment, the antibody was cleaved with papain prior to use (*34*). Where indicated, heparin was co-immobilized with antibody by adding heparin to a final

concentration of 10 μg/mL, after allowing antibody to bind to the surface of the ELISA tray for 30 min. After washing to remove unbound antibody, the wells were blocked for 1 h at room temperature using TB buffer (25 mM Tris-HCl, pH 7.4, containing 0.14 M NaCl, 1% BSA, and 0.01% Tween 20) before addition of solubilized EGFRs (1.2−2.5 μg total protein/well, unless otherwise stated) either in the presence or absence of ligand, as stated in the text. After allowing the receptor to bind for 3 h at 37 °C, unbound reagents were removed prior to addition of ^{125}I-labeled EGF (4 ng/mL, 2.6 MBq/μg). After a further 4 h incubation at room temperature, the wells were washed and dried before being punched from the tray; bound reactivity was determined using a gamma counter. Nonspecific binding was determined in the presence of 1 μg/well mEGF and subtracted from all data prior to analysis. Potencies were estimated by least-squares fitting to the logistic function $(a - d)/(1 + (x/c)^b) + d$.

To perform saturation binding analysis, the standard radioligand binding assay was performed using EGFRs which had been immobilized in the absence or presence of 330 nM EGF; radiolabeled EGF was varied in the range 0.05−20 nM while maintaining constant specific activity. Nonspecific binding was determined for each ligand concentration and subtracted from the corresponding data points before analysis. Data were analyzed by least-squares regression analysis and fitted to a bivalent receptor binding model as stated in the text.

For measurement of the dissociation of EGFRs from the immobilized EGFR1 antibody, EGFRs were immobilized in the presence EGF, as in the standard assay. After washing to remove unbound receptors, immobilized EGFRs were incubated in the absence or presence of competing EGFR1 monoclonal antibody (5−40 μg/well; this provided a ratio of competing:bound antibody in the range 25:1−200:1) for 2 h at 37 °C. After washing away any displaced receptor, radiolabeled ligand was added, and the assay was completed as in the standard protocol.

ELISA for Quantification of EGFR. EGFRs were immobilized onto MoAb EGFR1 as described above, except that the bound EGFR was quantified by addition of a polyclonal sheep anti-EGFR antibody (50 ng/well) for 1 h, followed by horseradish peroxidase conjugated anti-sheepIg antibody (1/1000) for 1 h. The antibody complex was visualized by addition of hydrogen peroxide with TMB as chromogen (*35*).

Comparative ligand binding affinities:competitive binding assays were performed as previously described (*24*), except that A431 cells were used at a density of 1×10^4 cells per well. Affinities were estimated by least-squares fitting to the logistic function $(a - d)/(1 + (x/c)^b) + d$.

RESULTS

It has previously been shown that bivalent antibodies to the EGFR are able to capture EGFRs as high affinity dimers which can be detected through subsequent binding of radiolabeled ligand (*15*). However, in these studies, a limiting concentration of cross-linking antibody was used to promote ligand-independent EGFR dimerization. This led us to determine whether we could use an excess of antibody and a limiting EGFR concentration to measure ligand-induced EGFR dimerization; under these conditions, we reasoned, the ligand-independent dimerization would be minimal and the presence of ligand would increase the probability of two EGFRs being simultaneously captured and tethered, one on each arm of the bivalent antibody.

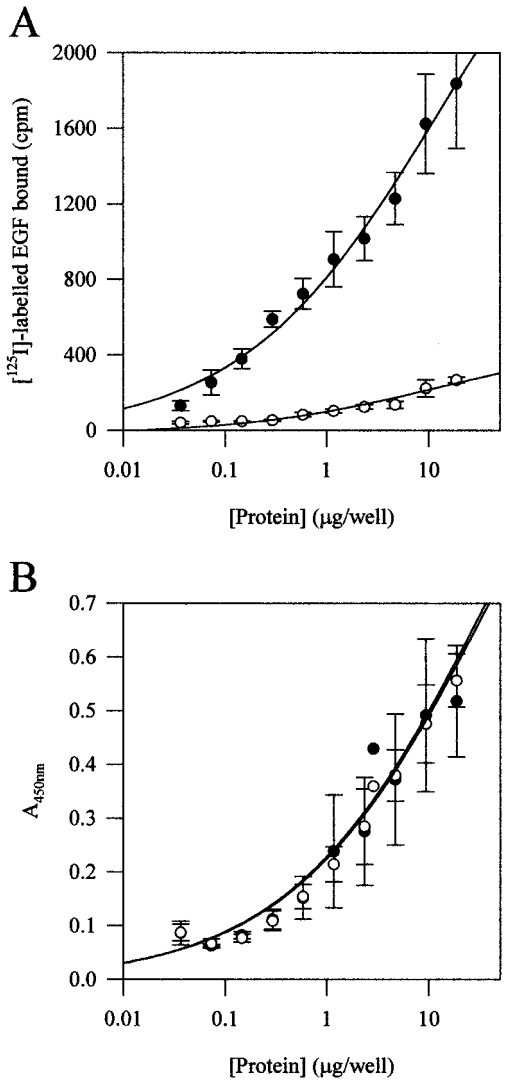

FIGURE 2: The enhancing effect of ligand pretreatment on binding of ^{125}I-labeled EGF by antibody-immobilized EGFRs. Serial dilutions of detergent solubilized EGFR were immobilized on MoAb EGFR1 in the presence (●) or absence (○) of 330 nM unlabeled EGF before being probed with (A) ^{125}I-labeled EGF or (B) sheep anti-EGFR, as described in Materials and Methods. Data are the mean ± SD ($n = 4$).

In our initial experiments, we found that when detergent-solubilized EGFRs were immobilized on a 96-well microtiter plate coated with the monoclonal anti-EGFR antibody EGFR1, subsequent binding of ^{125}I-labeled EGF was increased if the solubilized receptors had been immobilized in the presence of unlabeled EGF (Figure 2A). In either case, addition of unlabeled EGF after receptor immobilization had no further effect on the subsequent binding of radiolabeled ligand to these receptors (data not shown). The enhanced binding of ^{125}I-labeled EGF was not attributable to an increase in the amount of EGFR captured by the antibody, as control experiments showed that the quantity of immunoreactive EGFR remained the same irrespective of whether the receptor was immobilized in the presence or absence of ligand (Figure 2B). Although previous studies have shown that antibodies are able to induce receptor

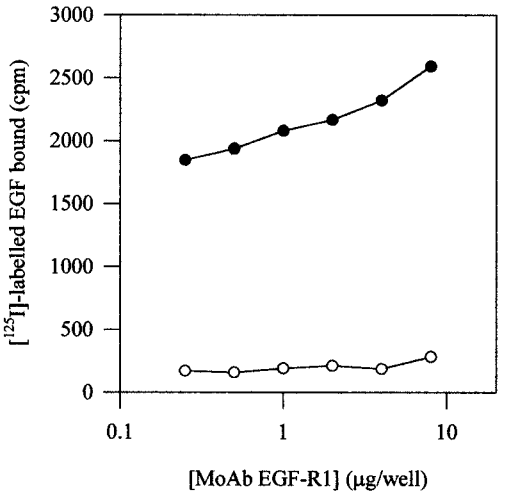

FIGURE 3: The effect of immobilizing antibody concentration on EGFR dimerization. Detergent-solubilized EGFRs were immobilized onto a range of concentration of MoAb EGFR1 in the presence (●) or absence (○) of EGF (330 nM) before being probed with ^{125}I-labeled EGF as described in Materials and Methods. Data are the mean of two independent observations.

dimerization through their bivalent nature (*15*), we observed little evidence for receptor dimerization in the absence of EGF over the range of antibody concentrations tested (Figure 3). While we also tested whether there was a requirement for antibody bivalency to stabilize the EGF-treated EGFRs as dimeric complexes, we failed to obtain significant binding of EGFRs to Fab fragments of the EGFR1 monoclonal antibody (data not shown), thus precluding further analysis.

To further characterize the ligand binding properties of the immobilized EGFRs, saturation binding studies were performed on EGFRs immobilized in the presence or absence of EGF (Figure 4A). Preliminary analysis of the data according to a one-site model of binding (data not shown) indicated that the enhanced association of ^{125}I-labeled EGF arose from a change in the number as well as the affinity of ligand binding sites, each by approximately 5−7-fold. This finding is apparently at odds with the results of the EGFR ELISA (Figure 2B) which demonstrates that the amount of receptor immobilization is independent of the ligand concentration. Although failure to detect these additional ligand binding sites in the ELISA might be explained by blockade of the antibody binding site on receptor dimerization, this seemed unlikely as the secondary antibody used in the EGFR ELISA was polyclonal and capable of recognizing many different epitopes. An alternative explanation is simply that the ligand binding assay only detects EGFR dimers, and that ligand bound to the much lower affinity monomeric EGFRs is removed during the final washing protocol. According to this scheme, the total measured binding of ligand arises from the formation of RRL and LRRL:

$$\mathrm{RR} + \mathrm{L} \rightleftharpoons \mathrm{RRL} \text{ (equilibrium constant } K_2)$$

$$\mathrm{L} + \mathrm{RRL} \rightleftharpoons \mathrm{LRRL} \text{ (equilibrium constant } K_3)$$

Given that antibody-immobilized EGFRs were found to have a very low dissociation rate with very little receptor being displaced, even in the presence of a 200-fold excess of EGFR1 antibody (Figure 5), we assume that the receptors are not released during the radioligand binding step and that

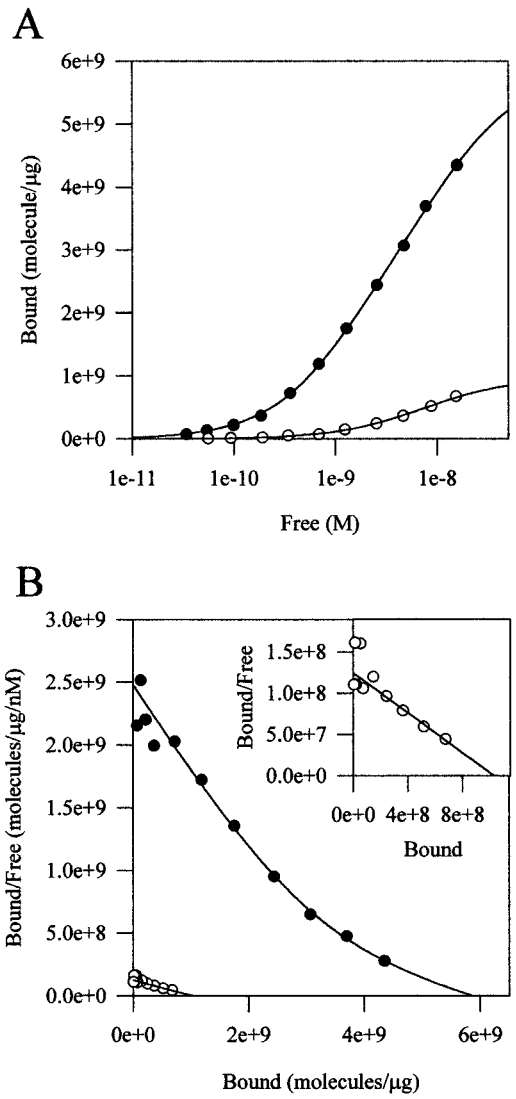

FIGURE 4: Saturation binding profiles for antibody-immobilized EGFR. Detergent-solubilized EGFRs were immobilized onto MoAb EGFR1 in the absence (○) or presence (●) of 330 nM EGF before being probed with a range of ^{125}I-labeled EGF concentrations as described in the Materials and Methods. Data are the mean of triplicate determinations and are representative of two independent experiments; (B) shows a Scatchard plot of the data presented in (A). Curves were fitted according to a bivalent receptor binding model as detailed in the text.

the concentration of RR remains constant following the immobilization protocol. Thus the equation for bound tracer can be derived as

$$[\mathrm{Bound}] = K_2[\mathrm{L}][\mathrm{RR}]_t(1 + 2K_3[\mathrm{L}])/(1 + K_2[\mathrm{L}](1 + K_3[\mathrm{L}]))$$

where $[\mathrm{RR}]_t$ is the concentration of immobilized dimeric receptors and [L] is the free tracer concentration. When this equation is fitted to the experimental data for receptors immobilized in the presence of EGF, we obtained values for K_2, K_3, and $[\mathrm{RR}]_t$ (Figure 4B, Table 1) which were in good agreement with those previously published for the binding of EGF to the extracellular domain of the EGFR (*16*). Thus, we suggest that our assay depends on the ability of ligand to increase the probability of immobilizing two EGFRs together, one on each arm of the bivalent EGFR1

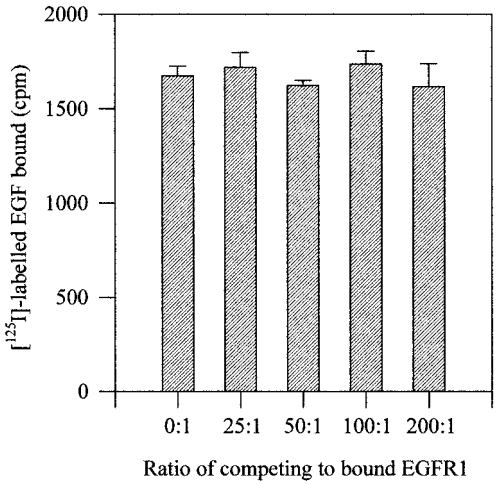

FIGURE 5: Measurement of the reversibility of EGFR immobilization by the EGFR1 monoclonal antibody. EGFRs were immobilized onto EGFR1 monoclonal antibody-coated ELISA trays in the presence of EGF (330 nM) according to the standard protocol. After washing to remove unbound EGFRs, EGFR1 antibody was introduced into the wells to measure displacement of EGFRs from the surface-bound antibody as detailed in Materials and Methods. After washing to remove displaced EGFRs, bound receptors were quantified with ^{125}I-labeled EGF. Data shown are the mean ± SE ($n = 3$).

Table 1: Equilibrium Constants for EGF Binding to Antibody-Immobilized EGFRs[a]

	K_2 (M^{-1})	K_3 (M^{-1})	Receptor (molecules/μg)
expt 1; −EGF	$1.2 \pm 0.1 \times 10^8$	0[b]	$10.4 \pm 0.4 \times 10^8$
expt 1; +EGF	$8.4 \pm 0.5 \times 10^8$	$7.3 \pm 1.9 \times 10^7$	$60 \pm 30 \times 10^8$
expt 2; −EGF	$2.8 \pm 0.5 \times 10^8$	0[a]	$5.2 \pm 0.4 \times 10^8$
expt 2; +EGF	$12 \pm 1 \times 10^8$	$10 \pm 7 \times 10^7$	$36.8 \pm 3.6 \times 10^8$
Lemmon et al. (*16*)	$5 \times 10^8 - 5 \times 10^{10}$	$\approx 7.5 \times 10^7$	−

[a] Estimates for K_2, K_3, and the number of binding sites were determined by fitting data for binding of ^{125}I-labeled EGF to antibody-immobilized EGFR, as described in Results. [b] Binding did not show obvious curvilinearity and was instead fitted to a simple monovalent binding model.

MoAb. As the enhancing effect of EGF on subsequent binding of ^{125}I-labeled EGF was concentration dependent and saturable ($EC_{50} = 2$ nM) (see Figure 8), we conclude that our assay enables direct quantification of ligand-induced dimerization.

When we compared the ability of the other cognate ligands for the EGFR to reproduce the effect of EGF in our binding assay, we found that mEGF, hEGF, TGFα, and HB-EGF all had comparable activities, while BTC was somewhat less effective (Figure 6). In contrast, AR_{1-84} was completely ineffective and was no better than acidic fibroblast growth factor (aFGF) which was included as an irrelevant ligand control.

Unlike the other EGFR ligands, AR_{1-84} is unusual because it has a truncated C-terminal tail that lacks the conserved leucine residue, and this results in a lowering of its affinity for EGFR by approximately 2 orders of magnitude (*31*). Thus, we compared the activity of AR_{1-84} with a C-terminally extended form of AR (AR_{1-90}, possessing a methionine at the conserved position) and with two recombinant EGFs with modifications to the C-tail (L47A, a mutant form of hEGF in which the conserved leucine in the C-tail

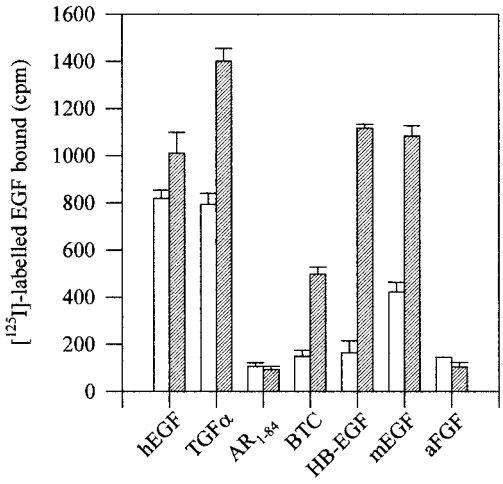

FIGURE 6: Comparison of the ability of EGF ligand family to promote EGFR dimerization. Detergent-solubilized EGFRs were immobilized onto MoAb EGFR1 in the presence of 3 (open bars) or 30 ng/well (hatched bars) of mEGF, hEGF, TGFα, BTC, AR_{1-84}, HB-EGF or aFGF before being probed with ^{125}I-labeled EGF as described in Materials and Methods. Data shown are mean ± SD ($n = 3$).

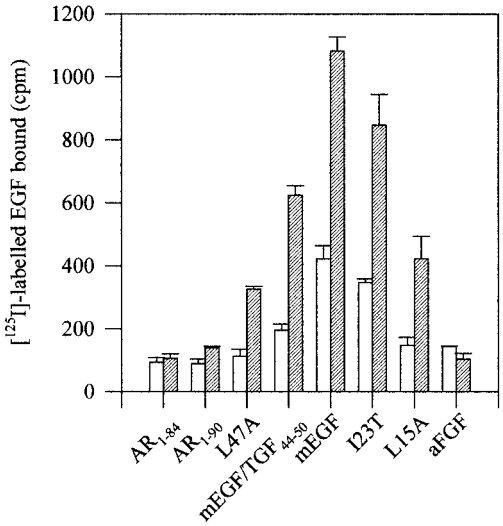

FIGURE 7: Comparison of the ability of AR and several mutant ligands to promote EGFR dimerization. Detergent-solubilized EGFRs were immobilized onto MoAb EGFR1 in the presence of 3 (open bars) or 30 ng/well (hatched bars) of AR_{1-84}, AR_{1-90}, L47A mEGF/TGF_{44-50}, mEGF, I23T, L15A, or aFGF before being probed with ^{125}I-labeled EGF as described in Materials and Methods. Data are the mean ± SD ($n = 3$).

is mutated to alanine and the chimera mEGF/$TGF\alpha_{44-50}$ which comprises mEGF where the C-terminal tail is substituted with that of TGFα) (Figure 7). Furthermore, we also tested two other mutant EGFs (I23T and L15A) to investigate the effect of mutations at sites in the main body of the growth factor (Figure 7). With the exception of AR, the concentration dependence of all these ligands in the dimerization assay closely paralleled their affinity, as determined by competitive binding (Figure 8, and Table 2).

The failure of AR to generate a response in accordance with its affinity suggested that some property of the ligand limits its ability to dimerize the EGFR. This cannot easily be attributed to the presence of the ~30 residue heparin binding domain per se, as HB-EGF also possesses a similar domain, but is equipotent with EGF. However, it has been

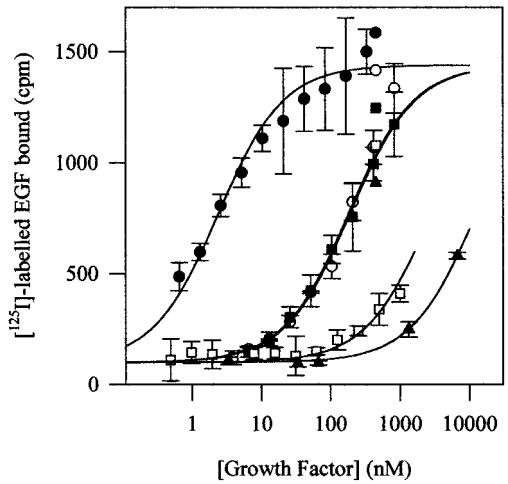

FIGURE 8: Concentration dependence of ligand pretreatment on binding of ^{125}I-labeled EGF by antibody-immobilized EGFRs. Detergent-solubilized EGFRs were immobilized onto MoAb EGFR1 in the presence of a range of concentrations of unlabeled ligand before being probed with ^{125}I-labeled EGF as described in Materials and Methods. Symbols correspond to hEGF (●), L47A (○), L15A (■), AR_{1-90} (□), and AR_{1-84} (▲). Data are the mean ± SD (n = 3).

Table 2: Summary of the Binding Affinities and Corresponding Dimerization Potencies for Selected Ligands[a]

ligand	competitive binding assay IC_{50} (nM)	dimerization assay EC_{50} (nM)
hEGF	3.0 ± 0.15	2.5 ± 0.3
I23T	20 ± 2	>3 and <30[b]
L15A	100 ± 15	190 ± 20
L47A	150 ± 10	170 ± 20
AR_{1-84}	350 ± 15	12000 ± 2000
AR_{1-90}	110 ± 10	3000 ± 1000

[a] Estimates of IC_{50} and EC_{50} values were obtained by fitting dose−response curves as described in Materials and Methods. [b] Estimated from Figure 7.

demonstrated that binding of AR to the EGFR is promoted by its association with heparan sulfate proteoglycans (HSPGs) (*36*). This led us to determine whether the kinetics of AR-induced EGFR dimerization is unfavorable because of the absence of a stabilizing effect from HSPGs. To mimic the presence of cell surface HSPGs, we co-immobilized MoAb EGFR1 together with heparin before performing our standard assay. As shown in Figure 9, localization of heparin onto the surface of the ELISA tray enhanced the capture of EGFRs by AR_{1-90}. In contrast, soluble heparin added together with the amphiregulin during the capture of EGFR had no significant effect. In control experiments, soluble or immobilized heparin had no effect on the ability of EGF to enhance EGFR dimerization (data not shown).

DISCUSSION

In this work, we describe a simple 96-well assay to measure ligand induced EGFR dimerization and demonstrate its utility in probing structure−activity relationships. The basis of the assay lies in the increased probability of EGFRs being captured as dimers by a bivalent antibody when the receptors are immobilized in the presence of cognate ligand. Once immobilized, the antibody acts as a tether, retaining the receptor in its dimeric state for subsequent measurement of radiolabeled ligand. The washing protocol of the assay

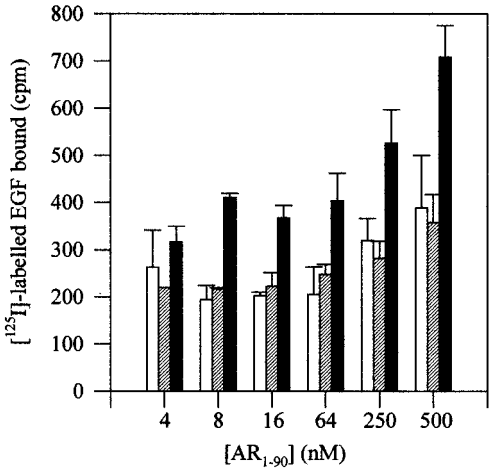

FIGURE 9: The influence of heparin on AR-induced EGFR dimerization. Detergent-solubilized EGFRs were immobilized onto MoAb EGFR1 in the presence of increasing concentrations of AR_{1-90}. The results show ^{125}I-labeled EGF binding for immobilization performed in the absence of heparin (open bars), in the presence of 10 μg/mL heparin in solution (hatched bars), or immobilized onto the surface of the ELISA tray together with EGFR1 as described in Materials and Methods (filled bars). Data are the mean ± SD (n = 3).

exploits the very low affinity of monomeric EGFRs for ligand such that tracer is only captured by the (higher affinity) EGFR dimers. Under the conditions used in our standard assay, we routinely observed a 5−7-fold enhancement of ^{125}I-labeled EGF binding if the EGFRs were immobilized in the presence of ligand. Saturation binding studies indicated that this was caused by an increase in the number of EGF binding sites as well as their affinity. The observed Scatchard plots support a model in which immobilized receptor dimers bind ligand at two nonequivalent sites with affinities that are in remarkable agreement with previously published parameters for EGF binding to EGFR extracellular domains (*16*).

The irreversible nature of the immobilization step (Figure 5) is crucial for the success of the assay; this ensures that captured receptors cannot redistribute during the subsequent radiolabeled ligand binding step, even though EGF is present to promote dimerization. This phenomenon was not peculiar to the EGFR1 antibody, as similar results were also obtained using EGF as the immobilizing ligand (data not shown). Although we have been unable to identify the precise cause of this unusually high-affinity interaction, we speculate that the hydrophobic nature of the ELISA tray caused redistribution of EGFRs from the detergent micelles onto the antibody-coated surface. Even though gel filtration experiments indicated that the detergent micelles contain between two and eight EGFRs (data not shown), we suggest that this redistribution leaves only one receptor molecule per antibody binding site.

We attribute the binding of ^{125}I-labeled EGF to receptors immobilized in the absence of ligand on chance circumstances in which captured receptors are sufficiently close for dimerization to take place when the tracer ligand is introduced. We speculate that the lower affinity observed for this population of receptor dimers may be due to spatial or orientational constraints that are not properly satisfied when two receptors are captured independently. Our analysis of the binding of tracer to these receptors did not show the same clear pattern of low- and high-affinity sites; however, this is

not surprising given the likely heterogeneous receptor population and technical difficulties in measuring such low-affinity interactions.

Of the native EGFR ligands, only AR and BTC showed any significant drop in their abilities to generate immobilized receptors with high binding capacity. However, several mutant ligands which were tested (L47A, L15A, I23T, and mEGF/TGFα_{44-50}) all showed lowered potencies which approximately followed their affinities. These mutations are widely separated, and they reflect the involvement of the majority of the growth factor in the binding and dimerization process. The parallel pattern of ligand affinity and dimerization potency suggests a mechanism where the processes of ligand-induced receptor dimerization approach an equilibrium even in the presence of the antibody. However, if receptor capture by antibody is fast relative to ligand−receptor interaction, ligand can only influence the outcome of the binding assay if receptors are frequently released and rebound after dimerization. However, this is clearly not the case (Figure 5). Therefore, we believe that it is more likely that ligand-assisted receptor dimerization is fast relative to antibody capture and that the receptor is captured in its dimeric state. This view is further supported by the observation that preincubation of receptor and ligand prior to their immobilization did not alter the subsequent binding of radiolabeled ligand (data not shown).

Of all the ligands tested, AR_{1-84} and AR_{1-90} were by far the least potent, being ∼12000 times and ∼3000 times less effective than EGF, respectively. According to the pattern observed for other low-affinity ligands, the extended form (AR_{1-90}) should have a potency similar to that of L15A or L47A. That AR_{1-84} and AR_{1-90} were both less effective in the dimerization assay than expected by affinity alone implies unique physical properties for these two growth factors. Given that the potency of L47A was reduced in parallel with its receptor binding affinity, the inability of AR to generate dimeric receptors in our assay is not directly related to the absence of a leucine in the tail of the growth factor. One possible explanation is that AR possesses a high dissociation rate from EGFR dimers compared with other ligands. Ligand−receptor association could take place in the same way as for other ligands; however, in the case of AR, we argue that the unusually high dissociation rate could lead to receptor dissociation before both receptors were tethered to the antibody. Thus antibody would capture fewer receptor dimers than expected. The experiments with heparin support the view that AR possesses anomalous binding kinetics; immobilized heparin markedly increased the potency of AR. Presumably the interaction with heparin increased the local concentration of AR at the surface of the well and/or decreased the rate of dissociation from the EGFR, thus facilitating dimerization and receptor activation. This observation also echoes the finding that HSPGs can enhance the ability of AR to stimulate cells (*36*), a mechanism which is undoubtedly important for the targeting of AR within tissues. Furthermore, it may also explain the very low potency of AR in some assays with cells possessing low numbers of EGFRs (*37*).

We have demonstrated that our EGFR dimerization assay can distinguish between good and poor ligands for the EGFR and thus represents a new method for routine screening of potential EGFR agonists. In its present form, this assay cannot distinguish between poor ligands which fail to bind to the receptor and those with a poor ability to generate receptor dimers (i.e., receptor antagonists). However, by introduction of a competing reference ligand during the immobilization step, ligand binding and receptor dimerization steps could be separated. Thus receptor antagonists would be detected as ligands which both reduced the efficacy of the reference ligand and which were poorly able to dimerize the receptor in their own right.

To date, the generation of EGFR ligand antagonists has not been particularly successful. However, this probably reflects the complexities of the ligand binding process, where high-affinity binding is associated only with receptor dimerization. Nevertheless, a precedent for the existence of such molecules is the *Drosophila* protein Argos (*38*), which functions as extracellular inhibitor of *Drosophila* EGFR (DER) preventing signal transduction via the ras/MAP kinase pathway (*39*). Even though Argos does not bind to the human EGFR, the structural similarity of the *Drosophila* EGFR suggests that suitable modifications to the Argos protein by genetic selection techniques (*40*) may generate ligands with antagonist properties. Furthermore, given the rapid advances in synthetic libraries, the scope for generation of entirely novel compounds is enormous. This assay represents an important advance which should enable the systematic screening of all of these molecules.

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BI972548X