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Ligand binding induces a conformational change in epidermal growth factor receptor dimers

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Abstract

The activation of the epidermal growth factor receptor (EGFR) kinase requires ligand binding to the extracellular domain (ECD). Previous reports demonstrate that the EGFR-ECD can be crystallized in two conformations – a tethered monomer or, in the presence of ligand, an untethered back-to-back dimer. We use Biosensor analysis to demonstrate that even in the monomeric state different C-terminal extensions of both truncated ($EGFR_{1-501}$)-ECD and full-length $EGFR_{1-621}$-ECD can change the conformation of the ligand-binding site. The binding of a monoclonal antibody mAb806, which recognizes the dimer interface, to the truncated $EGFR_{1-501}$-Fc fusion protein is reduced in the presence of ligand, consistent with a change in conformation. On the cell surface, the presence of erythroblastosis B2 (erbB2) increases the binding of mAb806 to the EGFR. The conformation of the erbB2: EGFR heterodimer interface changes when the cells are treated with epidermal growth factor (EGF). We propose that ligand induces kinase-inactive, pre-formed EGFR dimers and heterodimers to change conformation leading to kinase-active tetramers, where kinase activation occurs via an asymmetric interaction between EGFR dimers.

Keywords: *asymmetric tetramer, aggregation, kinase activation, structural models*

Abbreviations: *EGFR, epidermal growth factor receptor, ECD, extracellular domain*

Our understanding of the mechanism of activation of erbB receptor kinases is at a fascinating phase: the detailed three-dimensional (3D) conformations for many of the sub-domains (extracellular ligand-binding domains, trans- and juxtamembrane domains and the tyrosine kinase domains) have been determined (Garrett et al. 2002; Ogiso et al. 2002; Burgess et al. 2003; Garrett et al. 2003; Jorissen et al. 2003; Ferguson 2004; Leahy 2004; Bouyain et al. 2005; Ferguson 2008). The aggregation state of the receptors in the unligated and ligand-bound states is gradually becoming clearer (Moriki et al. 2001; Ichinose et al. 2004; Clayton et al. 2005; Lemmon 2009), but the mechanisms associated with ligand

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binding and kinase activation of the epidermal growth factor receptor (EGFR) require further clarification. Unless the EGFR is mutated, ligand binding is required for kinase activation; however, ligand binding is not required for EGFR dimer formation (Moriki et al. 2001; Ichinose et al. 2004; Clayton et al. 2005). The availability of the 3D structures for the extracellular domain (ECD) revealed the potential for significant ligand-induced conformational change (Garrett et al. 2002; Ogiso et al. 2002; Ferguson 2004; Leahy 2004). In particular, while 3D structures are not available for the full-length EGFR, models have been developed whereby low-affinity receptors (tethered by intramolecular interaction of domains II and IV) bind ligand and change shape to form a back-to-back dimer, where intermolecular interaction is mediated by the dimerization arms, resulting in activation of the intracellular tyrosine kinase (Burgess et al. 2003; Leahy 2004; Ferguson 2008; Lemmon 2009). Although these models are consistent with many experimental observations, the difference between the binding affinity of the soluble wild-type (wt) EGFR (tethered) (K_D: 200–500 nM) (Domagala et al. 2000; Elleman et al. 2001) and the K_D of low-affinity cell surface EGFR (1–10 nM) (Berkers et al. 1991) suggest a significant difference in the conformation of the ligand-binding site between the ECD in solution and the full-length EGFR on a cell surface. In solution, the K_D of an analogue of the EGFR-ECD ($sEGFR_{1-501}$) where most of domain IV had been removed, and therefore the ECD must be in the untethered conformation, is in the range of 1–15 nM (Elleman et al. 2001). The high-affinity state of the EGFR on the cell surface binds EGF with even higher affinity (20–50 pM). Furthermore, there is a strong correlation between the quantitative biological responses of cells to EGFR family ligands and the number of high-affinity receptor binding sites (K_D: 0.01–0.1 nM) (Walker et al. 1998a), suggesting that the untethered receptor, or receptor complexes facilitated by untethering, may represent the high-affinity sites both *in vitro* and on the cell surface, and that these sites are preferentially involved in EGFR signalling. At the time of writing, there are no reports of monomeric forms of sEGFR with affinities <15 nM (Elleman et al. 2001). However, several homo- and heterodimeric forms of the EGFR-ECD, e.g. $EGFR_{1-501}$-Fc (Adams et al. 2009), and $EGFR_{1-621}$-Fc (Jones et al. 1999), EGFR–erbB2-Fc (Jones et al. 1999) or EGFR–erbB3-Fc (Sarup et al. 2008) exhibit K_Ds for ligands in the range of 0.5–10 nM. Clearly, the close proximity of a second ECD can influence the structure of the binding site for the EGFR. Similar interactions have been demonstrated to occur on the cell surface, such that the affinity of the EGFR for its ligands is altered when erbB2 is present (Wilkinson and Staros 2002; Clayton et al. 2007; Li et al. 2012). A number of biophysical studies now support the concept that in the absence of ligand pre-formed EGFR dimers exist on the cell surface (Gadella and Jovin 1995; Moriki et al. 2001; Clayton et al. 2005; Hofman et al. 2010; Hsieh et al. 2010) and the addition of ligand stimulates the formation of higher order oligomers of the EGFR (Gadella and Jovin 1995; Clayton et al. 2005; Clayton et al. 2007; Clayton et al. 2008; Hofman et al. 2010).

Monoclonal antibodies have been extraordinarily useful probes for identifying functional regions of the EGFR-ECD as well as for detecting conformational changes in the receptor (Schmiedel et al. 2008; Schmitz and Ferguson 2009). In order to identify different conformational variants in the ECD of the EGFR, we have examined the binding of two antibodies mAb528 (Kawamoto et al. 1983) and mAb806 (Luwor et al. 2001; Mishima et al. 2001; Johns et al. 2002) to different forms of the EGFR in solution and on the cell surface. mAb528 binds to domain III blocking EGF binding (Kawamoto et al. 1983; Li et al. 2005), whereas mAb806 binds to an unusual epitope remote from the ligand-binding site but close to the dimerization surface (Johns et al. 2004; Garrett et al. 2009). mAb806 recognizes a conformation of the EGFR which is detected on some forms of the EGFR during ligand activation (Walker et al. 2004) and when it is overexpressed on tumour cells (Johns et al. 2002), but not on normal cells which express low levels of the receptor (Johns et al. 2002).

Although comparisons of binding kinetics for solution and cell surface interactions are complex (Adak et al. 2011; Pike 2012), the changes we observe in solution and on the cell surface for the binding of the anti-EGFR and anti-erbB2 antibodies are consistent with a model where activation requires a ligand-induced conformational change of an EGFR dimer on the cell surface. We propose that the ligand bound, back-to-back conformation of the EGFR dimer (Garrett et al. 2002; Ogiso et al. 2002) oligomerizes, inducing asymmetric dimer–dimer interactions (Groenen et al. 1997; Zhang et al. 2006) between the intracellular domains of the EGFR and consequential activation of the tyrosine kinase.

Experimental procedures

Antibodies used in this study: mAb806 (Jungbluth et al. 2003), mAb528 (Kawamoto et al. 1983; Sobol et al. 1987) and mAb3B10 (an antibody raised against the CR2-dimerization loop of the human ErbB2-ECD, see Supplementary Methods) were produced in the Biological Production Laboratory of the LICR in Melbourne, Australia. The ErbB2-specific antibodies Ab5 and Ab3 were purchased from Oncogene Research, La Jolla, CA, and trastuzumab (Carter et al. 1992; Baselga et al. 1998) was a kind gift from Dr

Terrence Johns, Monash University, Melbourne, Australia.

Production of EGFR analogues

The derivation of cell lines secreting sEGFR$_{1-501}$, EGFR$_{1-501}$-Fc and EGFR$_{1-621}$ and the purification of the respective proteins have been described previously (Elleman et al. 2001; Adams et al. 2009). Stable cell lines were grown in 15-cm tissue culture dishes for 5 days until confluent. Conditioned medium was collected and spun at 1500 rpm for 30 min and then filtered (Fast polyethersulfone (PES) Filter Unit 0.2 μm, Nalgene filtration products). The protein was purified using an AKTA FPLC using a mAb528 affinity column.

EGFR$_{1-621}$ fluorescent constructs

EGFR$_{1-621}$ was assembled using pEGFR513 (Elleman et al. 2001) and using the plasmid pEGFR comprising nucleotides 167–3970 of hEGFR (Ullrich et al. 1984). The forward primer was designed to amplify upstream of amino acid 513 to incorporate the unique enzyme site Psi1. The reverse primer was designed to incorporate amino acid 621 with Kpn1 and Age1 for in frame sub-cloning. The polymerase chain reaction (PCR) product was cloned into the pPCR-Script Amp vector (Stratagene). From here, it was sub-cloned into pEGFR513 using the Psi1 site and the Kpn1 site creating p621-pUC18. This was sub-cloned from this vector into pEGFP-N1 (Clontech, Mountain View, CA) via a Sal/Xho ligation and Age1 to create EGFR$_{1-621}$-GFP.

The YFP-linker-EGFR$_{1-621}$-GFP construct was made by cutting the GFP gene from the EGFR$_{1-621}$-GFP construct with Age1 and Not 1. The ends were blunted with Klenow and ligated creating EGFR$_{1-621}$-pEGFP-N1 (EGFR$_{1-621}$-GFP). yellow fluoresence protein (YFP) was amplified from the vector pEYFP-N1 (Clontech) and was inserted directly after the EGFR signal peptide with the first amino acid of EGFR starting after YFP. The final product was ligated into pPCR-Script (Stratagene). The resulting YFP-EGFR fragment was cloned into the amino terminal of the pEGFP-N1 vector via the pPCRScript BamH1/EcoR1 sites and Bgl11/EcoR1 in the vector to create YFP-EGFR$_{1-621}$-GFP. Three glycine linkers were added between the YFP and the first amino acid of EGFR using the Stratagene site directed mutagenesis kit according to the manufacturer's protocol to make the final construct YFP-linker-EGFR$_{1-621}$-GFP.

The RFP-EGFR$_{1-621}$-GFP construct was made by amplifying monomeric red fluoresence protein (RFP) by the same method used for YFP (mRFP was a kind gift from Dr Mark Prescott, Monash University, Australia). The RFP fragment was cloned into EGFR$_{1-621}$-GFP.

To assemble EGFR$_{1-621}$-albumin, the coding region (minus signal peptide) of a human albumin cDNA (clone HcCD00005810 from the DF/HCC DNA Resource Centre, Harvard University) was amplified by PCR to allow cloning in-frame behind EGFR$_{1-621}$ in the mammalian expression vector pME18s. A Flag epitope tag was included at the albumin C-terminus to allow purification of the fusion protein. 293 Freestyle (Invitrogen, Melbourne, Australia) cells, growing as suspension-adapted cultures (600 ml) in serum-free Freestyle^{TM} medium (Invitrogen), were transiently transfected with the pME18s-EGFR$_{1-621}$-albumin expression vector using polyethylenimine (Durocher et al. 2002). Culture supernatant was harvested after 7 days and Flag-tagged EGFR$_{1-621}$-albumin purified as described previously (Garrett et al. 2003).

Cell culture and DNA transfection

The constructs were transfected into human 293T fibroblasts maintained in DMEM supplemented with 10% FCS for transient transfection assays. The day before transfection, the cells were seeded at 2×10^5 cells per well in six-well tissue culture plates with 2 ml of media. Cells were transfected with 1 μg of plasmid DNA using FuGENE (Roche Molecular Biochemicals, Sydney, NSW, Australia) according to the manufacture's instructions. Supernatants and cells were harvested 48 h after transfection. Cells were lysed in 500 μl of lysis buffer (1% Triton X-100, 10% glycerol, 150 mM NaCL, 50 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), pH 7.4, 1 mM EGTA and complete protease inhibitor mixture Roche Molecular Biochemicals). Aliquots of supernatant and lysate were immunoprecipitated with mAb528 (Ludwig Austin) to recognize the ECD within the EGF-binding domain. Immune complexes were collected on Protein G Sepharose 4 Fast Flow beads (Amersham).

After the feasibility of protein expression was established via this transient transfection method, the HEK293 cells were then transfected with the successful constructs to produce stable cell lines which express the different fluorescent analogues of the EGFR. The following day standard media was replaced with media containing neomycin (G418) (Cellgro, Manassas, VA) at 1.5 mg/ml to select for colonies of stably transfected cells. After 4 weeks, isolated colonies were trypsinized and further diluted in 96-well plates. Stable cell lines were up-scaled from the 96-well plates as they became confluent. The best clones were selected for by their fluorescence and by the protein expression in the supernatant fluid by Western blotting.

Purification of $EGFR_{1-621}$ constructs

Except for the $EGFR_{1-621}$-Alb, all of the $EGFR_{1-621}$-proteins were purified by affinity chromatography using mAb528 coupled Protein G Sepharose. The antibody-coupled Sepharose was packed into a column (AKTA FPLC, GE Healthcare, Melbourne, Australia). The conditioned medium (adjusted to pH 8) was loaded on the column and eluted with 10 mm Glycine pH 2.5. The collected fractions were then neutralized and sized using a Superose 12 column (3.2/30) on a Pharmacia SMART micropurification system coupled to a diode array detector. The flow rate was 100 μl/min, and the column temperature was 25°C. Detection was at 280 and 215 nm and 100 μl samples were recovered. Collected proteins were quantitated on a NanoDrop ND-1000 spectrophotometer (Biolab, Melbourne, Australia) for further characterization and analysis.

Protein characterization

Fractions eluted from the superpose column were run on a 4–12% Bis-Tris gel (Invitrogen) and either Coomassie stained or silver stained to ensure homogeneity of fractions. Once this was established fractions were pooled and characterized by mass spectrometry. The bands were excised from a Coomassie-stained gel and mass spectrometry performed by Joint Proteomics Structure Facility (JPSF) laboratory at the Ludwig Institute for Cancer Research. Ultraviolet–visible scans of the protein showed the characteristic absorption and emission at different wavelengths of the different fluorophores and the concentration was calculated using the extinction coefficient (ExPASY protein tools) and the absorption of the protein at 280 nM.

Expression of EGFR, EGFR mutant and ErbB2 in BaF/3 cell line

BaF/3 cells expressing the wt or mutant EGFR have been described previously (Walker et al. 1998b). BaF cells expressing the Δ2-7EGFR are described in Luwor et al. (2004). Co-expression of ErbB2 with EGFR or its mutants was achieved by co-transfection in these cells of pRc/RSV vector containing ErbB2 cDNA with a hygromycin-resistance vector (pTK/Hyg). Selection was performed by culturing the cells in hygromycin (1 mg/ml) for 10 days. Viable cells were screened after this time for EGFR and ErbB2 expression by Fluoresence activated cell sorting (FACS) analysis using specific antibodies. Positive pools were subcloned by limiting dilution and re-screened for receptor expression. All cells were routinely passaged in Rosewall Park Memorial Institute (RPMI)/10% FCS/10% WEHI3B conditioned medium with G418 and hygromycin.

Modulation of antibody reactivity by pre-incubation

Cells expressing wtEGFR alone or in combination with ErbB2 were incubated with control medium (RPMI, 10% FCS, 20 μM phenylarsine oxide) alone (controls), or containing EGF(20 ng/ml), Ab5, trastuzumab, mAb3B10 (all at 10 μg/ml) in the appropriate combination. Our previous experience agrees with the published report (Hertel et al. 1985) that phenylarsine oxide inhibits EGFR internalization completely (Knutson et al. 1983; Hertel et al. 1985; Walker et al. 2004).

Cells were incubated with the antibodies at 37°C for 30 min to maximize the conformational changes. No internalization of the receptor occurs under these conditions.

Immunofluorescence analysis of receptor expression

Cells were collected by centrifugation and resuspended in FACS buffer (PBS/5% FCS/5 mM EDTA). Replicate samples were incubated with the appropriate antibodies, all at 10 μg/ml in FACS buffer, for 45 min at 4°C. After two washes with ice-cold FACS buffer the cells were incubated with the appropriate secondary antibody (Alexa-488 labelled anti-mouse Ig or Alexa 488-labelled anti-human Ig) for 40 min on ice. Cells were washed and resuspended in FACS buffer for analysis on a Becton & Dickinson FACScan. Background fluorescence was determined either by incubating the cells with an irrelevant antibody or by omitting the primary antibody. Peak and median channel fluorescence were determined using the Statistical program in CellQuest. For samples pre-incubated with mAb806 or mAb3B10, subtractive graphs (samples with pre-incubation – control samples) were used for the analysis.

Immunoprecipitation and immunoblotting

Cells were collected by centrifugation, washed in phosphate buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris, 150 mM NaCl, 5 mM ethylene diamine tetra acetic acid (EDTA), 1% Na Deoxycholate, 1% Triton X-100, 0.1% SDS) containing protease inhibitor cocktail (Roche) and phosphatase inhibitors (1 mM sodium orthovanadate and 5 mM NaF) on ice for 30 min. The lysates were centrifuged at 13,000 rpm for 15 min at 4°C, and the supernatants collected into fresh tubes. Lysates were incubated with anti-ErbB2 mAb Ab5 or anti-EGFR mAb528, both at 10 μg/ml, for 1 h at 4°C, followed by Sepharose-coupled Protein A (10 μl packed beads/reaction) for 40 min at 4°C with rotation. The beads were washed two times in RIPA buffer and once in PBS, then resuspended in reducing sample buffer and analysed by sodium dodecyl sulfate (SDS)/PAGE on 4–12% Bis-Tris gels (Invitrogen).

Gels were transferred to polyvinylidene fluoride (PVDF) membranes for immunodetection with either anti-EGFR antibodies (mAb806) or anti-ErbB2 antibodies (Ab3, Oncogene Research). Bands were visualized using Horse radish peroxidase (HRP)-coupled anti-mouse Ig antibodies and enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, England). For mitogen activated protein kinase (MAPK) activation cells were serum-starved in RPMI without addition for 2 h at 37°C prior to stimulation with EGF (100 ng/ml) or control medium for 10 min at room temperature. Cells were washed once in PBS on ice, and lysed directly in reducing sample buffer. Samples were separated on 4–12% Bis-Tris gels (Invitrogen), transferred onto Immobilon PVDF and probed with anti-phospho-MAPK antibody (phospho p44/42 MAPKinase, Cell Signaling) followed by HRP-coupled anti-rabbit Ig and ECL reagent (Amersham Pharmacia Biotech UK Ltd).

Biosensor experiments

Biosensor experiments were performed using a Biacore 3000 (GE Healthcare) instrument. Prior to immobilization the CM5 sensor chip was preconditioned with two injections of each of the following solutions, 100 mm HCl, 50 mM NaOH, 0.5% SDS and double distilled water (DDW). Following the preconditioning, the system was then primed a number of times before immobilization commenced. Recombinant human EGF (ProSpec-Tany Technogene Ltd, Ness-Ziona, Israel) and the mAbs 528 and 806 were immobilized onto separate channels of the CM5 sensor chip surface using amine coupling chemistry (*N*-hydroxysuccinimide and ethyl-Ni-dimethylaminopropyl-carbodiimide) at a flow rate of 10 μl/min. Flowcell 1 was blank derivatized.

To minimize mass transfer affects and rebinding, the sensor was set at a flow rate of 100 μl/min, sample volume injected was 140 μl and dissociation was 1000–3600 s. Unless indicated otherwise the protein concentrations used in the biosensor binding experiments were adjusted to 600 nM. Twofold serial dilutions down to 5 nM were made to generate the binding data.

Samples were diluted into Biacore running buffer [HBS: 10 mM HEPES (pH 7.4) containing 3.4 mM EDTA, 0.15 mM NaCl and 0.005% (v/v) Tween 20]. Regeneration of surfaces was performed at a flow rate of 100 μl/min, with two times 10 μl pulses of 10 mM Glycine pH 2.0. The surface was then injected with a short pulse of buffer to prevent carryover of the regeneration solution.

For the analysis of EGFR derivatives binding to immobilized antibodies (528 and 806) in the presence and absences of 500 nM EGF, 35 μl of samples were injected at a flow rate of 5 μl/min. The surfaces were regenerated as described previously.

Biosensor analysis

Sensor curves produced were analysed using a combination of Biaevaluation 4.1 and Scrubber 2.0 software. When analysing binding to immobilized EGF, kinetic constants were derived from the sensorgrams fitted using the two-state reaction (conformation change) binding model. The goodness of fit between experimental data and fitted curves was estimated by χ^2 analysis using the equation given by: $\chi = (\Sigma_n(\mathrm{rf}-\mathrm{rx})^{2)}/(n-p)$ where rf is the fitted value at a given point, rx is the experimental value at the same point, n is the number of data points and p is the number of degrees of freedom. Curves were also analysed by global fitting with a two-state reaction (conformational change) model where analyte (A) binds to ligand (B) (k_{a1} and k_{d1}). Complex AB changes to AB⋆, which cannot dissociate directly to A + B (k_{a2} and k_{d2}). The resulting affinity $(K(1/M) = (k_{a1}/k_{d1})\star(1 + k_{a2}/k_{d2})$.

Molecular modelling

Models of the EGFR kinase were developed using the coordinates deposited at the protein database (PDB) (http://www.pdb.org/pdb/home/home.do) (Zhang et al. 2006) for both the active EGFR kinase (PDB ID 2GS2) and inactive EGFR kinase (PDB ID 2GS7). Formation of the EGFR kinase dimers and tetramers was performed manually using PyMOL version 1.4.1 (http://www.pymol.org/).

Table I. EGFR derivatives binding to immobilized EGF.

Analyte	ka1 ($M^{-1}s^{-1}$) $\times 10^5$	k_{d1} (s^{-1}) $\times 10^{-3}$	K_{D1} (nM)	k_{a2} ($M^{-1}s^{-1}$) $\times 10^5$	k_{d2} (s^{-1}) $\times 10^{-3}$	K_{D2} (Nm)
$EGFR_{1-501}$	1.96	12	62	5.08	93.2	180
$EGFR_{1-501}$-FC	1.36	4.73	35			
$EGFR_{1-621}$	3.11	143	460	0.0856	9.8	1100
$EGFR_{1-621}$-albumin	3.68	262	710	0.0286	2.61	910
$EGFR_{1-621}$-GFP	0.282	0.974	35	3.53	328	930
RFP-$EGFR_{1-621}$-GFP	0.596	1.26	21	1.6	301	1900
YFP-linker-$EGFR_{1-621}$-GFP	0.599	1.91	32	1.26	148	1200

Results

Affinity of EGFR-ECD analogues for EGF

mAb806 and mAb528 recognize different conformations of the EGFR-ECD. mAb806 can be used to detect conformational changes in the receptor following ligand binding (Walker et al. 2004). We compared the binding profiles of both antibodies to the EGFR-ECD in solution. Analysis of EGFR derivatives binding to immobilized surfaces of EGF, mAb806 or mAb528 indicated significant changes in the conformational properties of the receptor when there was an N-terminal extension (YFP-EGFR or GFP-EGFR), a C-terminal truncation ($EGFR_{1-501}$) or a C-terminal extension (EGFR-GFP, EGFR-albumin or EGFR-Fc). All of the derivatives bound to the immobilized EGF (Table I, Supplementary Figure 1). As expected the full-length ECD ($EGFR_{1-621}$) had a low affinity, i.e. 460 nM (Table I). As reported by Elleman et al. (2001) the truncated ECD derivative $EGFR_{1-501}$ had considerably higher affinity (62 nM). Interestingly, the different C-terminal extensions to $EGFR_{1-621}$ had significant effects on the binding affinity of EGFR to the immobilized ligand. Thus, the addition of GFP to the C-terminus of the full-length $EGFR_{1-621}$-ECD ($EGFR_{1-621}$-GFP) or the addition of the immunoglobulin Fc domain to the C-terminus of $EGFR_{1-501}$ ($EGFR_{1-501}$-Fc) enhanced the receptor affinity binding significantly (Table I). Molecular weight analysis of $EGFR_{1-621}$-GFP, by sedimentation equilibrium, indicated that this analogue is a monomer (Supplementary Figure 2). Although the predicted molecular weight of the monomeric EGFR polypeptide chain is only 96,000 D, the large number of carbohydrate chains (Takahashi et al. 2008) increases the expected molecular weight to 130,000 D (Stroop et al. 2000), i.e. almost identical to the value calculated from the sedimentation equilibrium experiments, and yet the monomer has a high-affinity binding state for EGF (Table I, Supplementary Figure 1). The addition of albumin to the C-terminus of $EGFR_{1-621}$ made no significant difference to

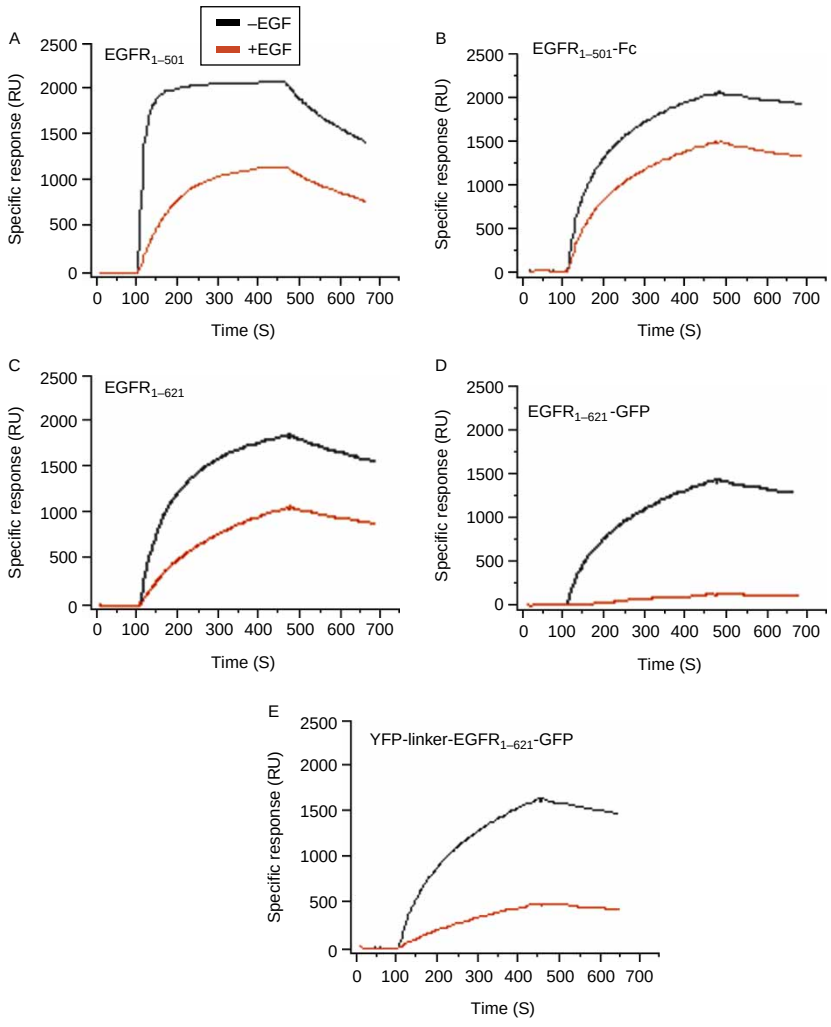

Figure 1. EGFR analogues binding to immobilized antibody mAb528 on the biosensor chip in the presence and absence of EGF: (A) $EGFR_{1-501}$, (B) $EGFR_{1-501}$-Fc, (C) $EGFR_{1-621}$, (D) $EGFR_{1-621}$-GFP and (E) YFP-linker-$EGFR_{1-621}$-GFP.

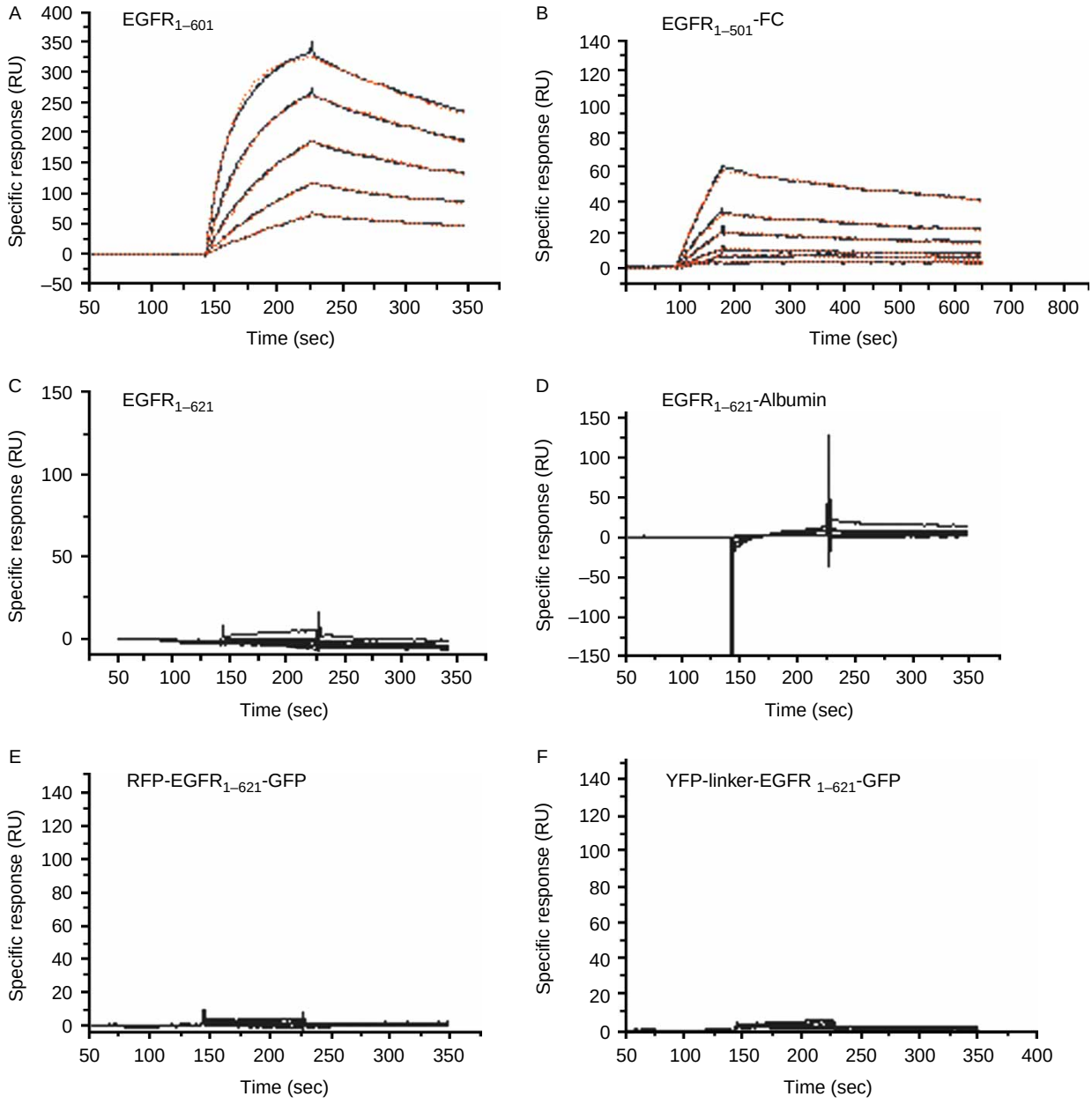

Figure 2. EGFR analogues binding to immobilized antibody mAb806 on the biosensor chip: (A) $EGFR_{1-501}$, (B) $EGFR_{1-501}$-Fc, (C) $EGFR_{1-621}$, (D) $EGFR_{1-621}$-albumin, (E) RFP-$EGFR_{1-621}$-GFP, and (F) YFP-linker-$EGFR_{1-621}$-GFP.

the binding affinity (Table I). Similarly, attaching N-terminal extensions (RFP- or YFP-) to $EGFR_{1-621}$-GFP had no significant influence on the affinity of the receptor to the EGF surface.

Antibody binding to EGFR-ECD analogues

Binding of mAb806 or mAb528 can be used to detect significant changes in the conformation of the soluble receptor analogues. Antibody mAb528 binds to domain III, competing for the major ligand contact surface. The binding of the all $EGFR_{1-621}$ derivatives to mAb528 was similar, i.e. K_D 10–20 nM (Supplementary Figure 3, Table II). This binding indicates that domain III is accessible in all of the analogues. Interestingly removal of the C-terminus ($EGFR_{1-501}$) increases the affinity for mAb528 (4.6 nM) and the dimeric form of the soluble, truncated receptor $EGFR_{1-501}$-Fc binds even more tightly (0.9 nM), suggesting a significant rearrangement of the ligand-binding domains. The on-rate for the reaction of $EGFR_{1-501}$-Fc was slightly reduced and the off-rate was significantly reduced when compared with truncated $EGFR_{1-501}$ or any of the full-length $EGFR_{1-621}$-ECD derivatives. As expected, binding of the EGFR derivatives to mAb528 was reduced in the presence of EGF (Figure 1).

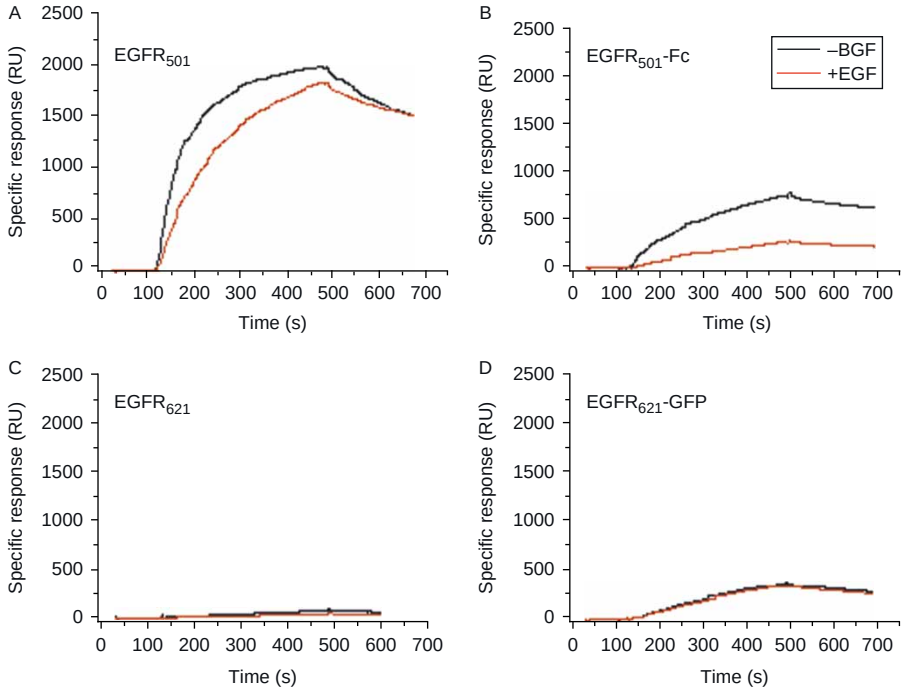

Figure 3. EGFR analogues binding to immobilized antibody mAb806 on the biosensor chip: in the presence and absence of EGF: (A) $EGFR_{1-501}$, (B) $EGFR_{1-501}$-Fc, (C) $EGFR_{1-621}$, and (D) $EGFR_{1-621}$-GFP.

mAb806 binds to an EGFR epitope remote from the ligand-binding site. When the wt receptor is expressed at low levels (<100,000 receptors/cell) mAb806 cannot detect this cryptic epitope (Garrett et al. 2009), however, if the receptor is truncated at the N-terminus (Δ2-7EGFR), overexpressed or mutated such that the dimerization arm no longer fits into its binding pocket during ligand binding, the epitope for mAb806 is exposed. The tethered conformations of the EGFR, e.g. $EGFR_{1-621}$ do not bind mAb806 (Figure 2). Even when extended at the N- and C-terminus the full-length EGFR-ECD (RFP-$EGFR_{1-621}$-GFP) fails to bind mAb806. However, removal of domain IV from the EGFR-ECD ($EGFR_{1-501}$) allows mAb806 binding (Figure 2A). The binding of $EGFR_{1-501}$ to mAb806 is not changed significantly in the presence of ligand (Figure 3A). However, when $EGFR_{1-501}$ is dimerized ($EGFR_{1-501}$-Fc) binding to mAb806 is reduced significantly in the presence of ligand (Figure 3B), indicating that ligand binding to domain III, in the context of the Fc-associated dimerization, results in a significant conformational change at this dimerization interface.

ErbB2 induces a change in the binding of mAb806 to the EGFR

mAb806 can also be used to detect conformational changes in homo- and heterodimers of cell surface EGFR. As mentioned earlier, when the EGFR is expressed on the cell surface at normal levels, mAb806 binding is <10% of the mAb528 binding. However, in the presence of erbB2, there is a fourfold increase in mAb806 binding to cell surface EGFR (Figure 4), implying a new conformation for the unligated EGFR–erbB2 heterodimer which cannot be completely tethered or back-to-back (where the mAb806 epitope would be hidden). After binding ligand the

Table II. EGFR derivatives binding to immobilized antibodies.

Antigen	Immobilized Surface	k_a ($M^{-1}s^{-1}$) $\times 10^4$	k_d (s^{-1}) $\times 10^{-4}$	K_D (nM)	χ^2
$EGFR_{1-501}$	mAb528	46	21	4.6	483
$EGFR_{1-501}$-Fc	mAb528	4.7	0.41	0.9	17
$EGFR_{1-621}$	mAb528	4.2	7.8	19	29.3
$EGFR_{1-621}$-albumin	mAb528	7.1	7.1	10	108
$EGFR_{1-621}$-GFP	mAb528	4.9	6.9	14	31.6
RFP-$EGFR_{1-621}$-GFP	mAb528	4.7	6.5	14	21.2
YFP-linker-$EGFR_{1-621}$-GFP	mAb528	4.1	5.6	14	30.7
$EGFR_{1-501}$	mAb806	16	28	17	9.18
$EGFR_{1-501}$-Fc	mAb806	1.2	7.7	63	0.301

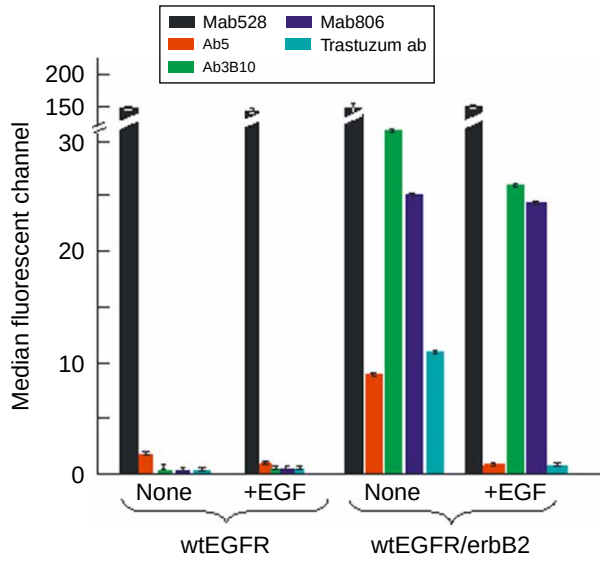

Figure 4. Fluorescence-activated cell sorting analysis of the effects of erbB2 and/or EGF on the binding of antibodies to wtEGFR and erbB2 expressed on Baf/3 cells. Cells expressing wtEGFR only or cells expressing both wtEGFR and erbB2. Antibodies used for the analysis: anti-EGFR mAb806; anti-erbB2: mAb3B10 – against the dimerization arm, mAb5 – raised against the ECD (not conformational) and trastuzumab – recognizes ECD IV.

EGFR–erbB2 heterodimer loses its ability to bind mAb806 and mAb3B10, suggesting a further conformational change, presumably towards the ligand-bound back-to-back conformation. The presence of erbB2 induces a higher proportion of the EGFR to expose the mAb806-binding site and most of the EGFR to bind ligand with high affinity ($K_{D1} = 0.3$ nM) (Table III). However, it should be noted that the affinity measurements made with fluorescent-EGF are not sensitive enough to quantitate pM binding with accuracy; Scatchard analysis of EGF binding using radiolabelled-EGF allows detection of high-affinity binding and of the changes induced by co-expression of ErbB2 (see Supplementary Figure 4). The change in mAb806 binding to the EGFR:erbB2 heterodimer induced by EGF is not due to heterodimer dissociation as erbB2 immunoprecipitates from Baf/3 cells, expressing EGFR and erbB2, contain the same amount of EGFR whether EGF is present or not (Figure 5). In the presence of EGF, the reduced binding of mAb806 to the cell surface EGFR:erbB2 heterodimer is analogous to the reduced binding of mAb806 to the $EGFR_{1-501}$-Fc analogue (Figure 3) or to the cell surface wtEGFR (Figure 4) in the presence of ligand.

mAb3B10 was raised to an epitope in the erbB2 CR1 dimerization arm, and normally recognizes a sub-population of ErbB2 where the epitope is exposed (Supplementary Figure 5). The mAb3B10 epitope is expected to be hidden during active, back-to-back dimerization of erbB2 or heterodimerization with the EGFR. When the wtEGFR and wtEGFR/erbB2-Baf/3 cells are exposed to ligand the binding of both mAb806 and mAb3B10 are abolished, reflecting a re-orientation of the heterodimers. It is also possible that the ligand-induced oligomerization (Clayton et al. 2005) inhibits access of these antibodies to their epitopes. In contrast, the binding of trastuzumab or Ab5 (which recognize non-conformational epitopes on erbB2) is not affected significantly in the presence of EGF (Figure 4). Conversely, when the cells are pre-incubated with mAb3B10, the mAb806 epitope on the EGFR is more accessible (Figure 6). Presumably, the blocking of the erbB2 CR1 dimerization arm by mAb3B10 disrupts the back-to-back conformation of heterodimers. This is also reflected in a reduction in the effect of ligand on the accessibility of mAb806 to the EGFR in the EGFR–erbB2 heterodimer (Figure 6).

Pre-treatment of wtEGFR/erbB2-Baf/3 cells with mAb3B10 decreases trastuzumab binding, possibly through steric hindrance. When these cells are pre-treated with trastuzumab, binding of mAb806 to the EGFR is abolished completely (Figure 6), while binding of mAb3B10 to ErbB2 is enhanced. Since the epitope for trastuzumab resides in the CR2 domain, antibody binding to this region would be expected to hinder CR1/CR2 interactions, thus freeing the CR1 for binding to 3B10. These experiments clearly show that heterodimerization with ErbB2 alters the conformation of the EGFR, and that the conformation of each member of the heterodimer is further regulated by EGF binding. By inference, there must be constitutive formation of heterodimers and ligand-induced activation of the heterodimers.

Cell surface EGFR:erbB2 heterodimers require ligand to activate the kinase

When the EGFR and erbB2 are co-expressed on cells, e.g. Baf/3, in the absence of ligand, there is a significant effect on the proportion of high-affinity ($K_{D1} = 0.3$ nM) and low-affinity ($K_{D2} = 2$ nM) EGFR (as determined by the EGF-binding kinetics, see Table III and Supplementary Figure 4). It is clear that the two receptors interact constitutively, as erbB2 immunoprecipitates from cells expressing erbB2

Table III. Kinetic analysis of binding of FITC-labelled-EGF to EGFR expressed on Baf/3 cells in the presence or absence of erbB2.

Parameter	wtEGFR	wtEGFR/erbB2
R^1: k_{on1} ($10^6 M^{-1} s^{-1}$)	9	3
k_{off1} ($10^{-3} s^{-1}$)	2	1
K_{D1} (nM)	0.2	0.3
R^1 (%)	13	97
R^2: k_{on2} ($10^6 M^{-1} s^{-1}$)	2	6
k_{off2} ($10^{-3} s^{-1}$)	2	12
K_{D2} (nM)	1	2
R^2 (%)	87	3

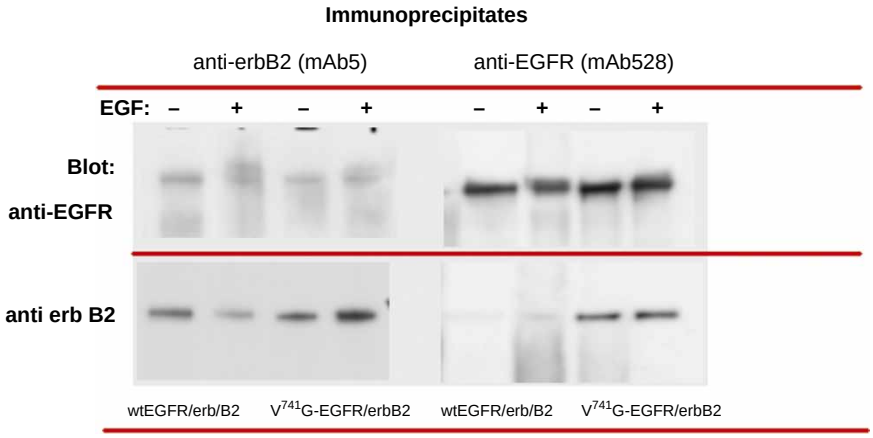

Figure 5. EGFR and erbB2 form a complex when co-expressed on Baf/3 cells. The $V^{741}G$ mutation in the EGFR kinase domain stabilizes the EGFR/erbB2 interaction.

contain significant levels of the EGFR (Figure 5). Interestingly, an EGFR analogue with an inactive kinase domain (EGFR-$V^{741}G$) interacts more strongly with erbB2 and the erbB2 is easily precipitated with the EGFR antibodies (Figure 5). Despite the presence of heterodimers, in the absence of ligand, there is no detectable signalling from the wtEGFR/erbB2 complex (Figure 7). This implies that dimerization alone is insufficient to activate the receptor complex.

Although the truncated Δ2-7EGFR elicits a weak, constitutive MAPK signal in the absence of ligand, in the presence of erbB2 constitutive signalling from Δ2-7EGFR is enhanced significantly (Figure 7). Signalling from the Δ2-7EGFR/erbB2 complex is not dependent on the presence of ligand. Truncation of the N-terminus of the EGFR-ECD must be sufficient to allow the kinase domains of the truncated Δ2-7EGFR/erbB2 complex to adopt an activated configuration. Similarly, signalling from the inactive EGFR mutant ($V^{741}G$) is not stimulated by ligand; however, the EGFR-$V^{741}G$/erbB2 complex has detectable signalling and is very responsive to ligand. Ligand fails to activate the kinase-defective EGFR-$K^{721}R$ analogue, however, the EGFR-$K^{721}R$/erbB2 complex is activated by EGF (Figure 7). Although it is impossible to discriminate between EGFR and ErbB2 kinase activation in cells co-expressing kinase competent EGFR constructs, it is clear that kinase-negative EGFR can activate the ErbB2 kinase in a ligand-dependent manner.

Collectively, these results support the presence of pre-formed heterodimers in the absence of ligands, and that binding of an EGFR ligand is required for the activation of the wtEGFR/erbB2 receptor complex.

Discussion

The mechanism of activation of the EGFR by its ligand has been the subject of considerable experimentation over many years (Schlessinger 2002; Jorissen et al. 2003; Klein et al. 2004; Dawson et al. 2005, 2007; Ferguson 2008). The details of ligand binding to cell surfaces and even to biosensor surfaces can be difficult to interpret (Macdonald and Pike 2008; Pike 2012). The stability of ligands and/or receptors, the valency of the reagents, aggregation of receptors and/or ligands and conformational changes combine to confound the development of models to explain much of the data. In particular, where antibodies or receptors are bivalent (e.g. the mono-

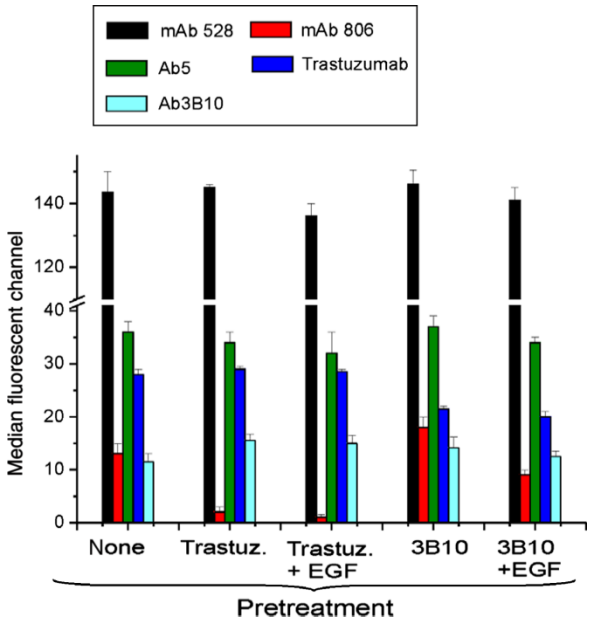

Figure 6. Fluorescence-activated cell sorting analysis of the effects of EGFR family antibody with or without EGF pre-treatment on the binding of domain-specific antibodies to EGFR and erbB2 expressed on Baf/3 cells. The wtEGFR/erbB2_Baf/3 cells were pre-treated for 30 min with control medium (RPMI, 10% FCS, 20 μM phenylarsine oxide) or the indicated antibody/EGF combination (10 μg/ml) for 30 min at 37°C. Trastuzumab but not mAb3B10 (the antibody which binds to the erbB2 dimerization arm) blocks the binding of mAb806 to Baf/3 cells expressing EGFR and erbB2.

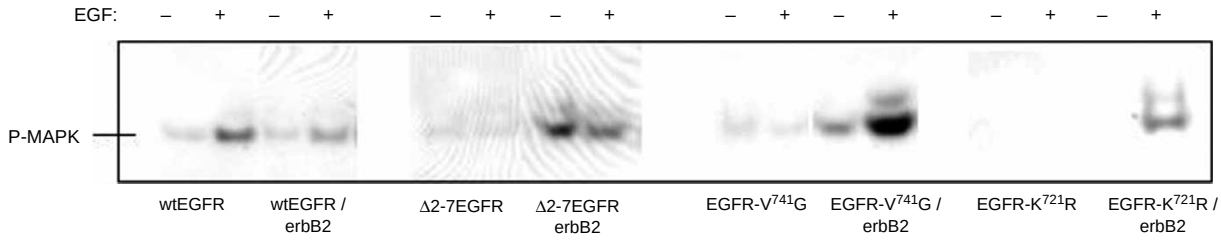

Figure 7. ErbB2 influences the ability of EGFR analogues to activate MAPK in response to EGF. Baf/3 cells expressing different EGFR/erbB2 receptor combinations were stimulated with EGF (100 ng/ml, +) or control medium (−) for 10 min and the cell extracts were analysed by Western blotting for phospho-MAPK. Although erbB2 has little apparent influence on the activity of the wtEGFR, it amplifies the constitutive effect of the Δ2-7EGFR on MAPK activation and increases the ability of V^{741}G-EGFR and K^{721}R-EGFR to activate MAPK in response to EGFR.

clonal antibodies or the $EGFR_{1-501}$-ECD-Fc) the apparent binding affinities can be influenced by valency. The expected back-to-back orientation of the EGFR dimer analogues used in this report does not allow both domains of the same antibody to bind to receptor dimers. However, at the biosensor coating density (antibody density $= 0.9\,ng/mm^2$) it is possible that a divalent EGFR analogue (e.g. $EGFR_{1-501}$-Fc) could bind to two separate antibodies. This interaction with two receptors might account for the decrease in the off-rate (thus an increase in apparent affinity of $EGFR_{1-501}$-Fc for mAb528). However, the affinities of $EGFR_{1-621}$, $EGFR_{1-621}$-albumin and $EGFR_{1-621}$-GFP for mAb528 are essentially the same (Table II). The monomeric molecular weight from the sedimentation equilibrium experiments and the 10-fold increase in affinity of the $EGFR_{1-621}$-GFP analogues for EGF, suggest to us that the increased affinity is likely to be associated with a conformational change to the EGF-binding region rather than valency changes associated with dimerization of these analogues. The discovery of two significantly different structures for the EGFR-ECD and the tethered forms of the ECD for other EGFR family members (erbB3 and erbB4) (Bouyain et al. 2005) as well as the requirement for asymmetric association of at least two intracellular domains for kinase activation (Groenen et al. 1997; Zhang et al. 2006; Bae and Schlessinger 2010) and the recent data on the oligomerization state of the EGFR in the presence and absence of ligand (Gadella and Jovin 1995; Moriki et al. 2001; Clayton et al. 2005, 2007, 2008; Hofman et al. 2010; Lu et al. 2010), require new models for the ligand-mediated activation of signalling from EGFR family.

Our new data on the EGFR both in solution and on the cell surface need to be considered along with other kinetic and cell surface measurements (Bedrin et al. 1997; Ozcan et al. 2006; Alvarado et al. 2010; Kozer et al. 2010, 2011a; 2011b; Chen et al. 2011). In solution the EGFR-ECD ($EGFR_{1-621}$) structure appears to be tethered, however, ligand can induce a conformational change (Greenfield et al. 1989), albeit at high concentrations (200–500 nM); ligand-induced dimerization of the free EGFR-ECD in solution also requires high (unphysiological) concentrations of ligand and receptor (~10 μM) (Rijken et al. 1991; Walker and Burgess 1991). In this context, it is interesting to note that specific C-terminal extensions to the soluble forms of the EGFR-ECD can influence the conformation of the ligand-binding domains (e.g. $EGFR_{1-621}$-GFP and Kozer et al. 2010), whereas other C-terminal extensions ($EGFR_{1-621}$-albumin) have no long-range effects on ligand binding. Our results indicate that the wtEGFR-ECD-GFP is monomeric (Supplementary Figure 2) and high affinity (Figure 1), i.e. untethered. Clearly, the nature of the C-terminal extension is important. Given that the cell surface EGFR in *Drosophila melanogaster* is untethered (Alvarado et al. 2009), and that even the low-affinity EGFR on the cell surface has 100-fold higher affinity than $EGFR_{1-621}$ (Elleman et al. 2001; Schlessinger 2002), it is interesting to consider the possibility that a small (<10% of the cell surface EGFR), but significant proportion of the cell surface wtEGFR is untethered (Supplementary Figure 6). These untethered EGFR appear to be high-affinity dimers which are able to interact with mAb806. In the presence of erbB2, the wtEGFR transitions from ~10% of the high-affinity state to 97% of the wtEGFR being in the high-affinity (300 pM) state. The higher affinity of the wtEGFR in the wtEGFR:erbB2 complex suggests that the EGFR-ECD is untethered. When ligand binds to either the heterodimer or the high-affinity homodimer a further conformational transition occurs, leading to the back-to-back dimer state and the masking of the mAb806 and mAb3B10 epitopes.

In the absence of ligands, both monomers and dimers of the wtEGFR are found on the cell surface (Gadella and Jovin 1995; Moriki et al. 2001; Clayton et al. 2005; Chung et al. 2010). However, we now need to consider the possibility that the tethered and untethered conformations of the ECD might be in dynamic equilibrium for both monomers and preformed dimers. The mAb806 conformational probe does not recognize the tethered form of the ECD of

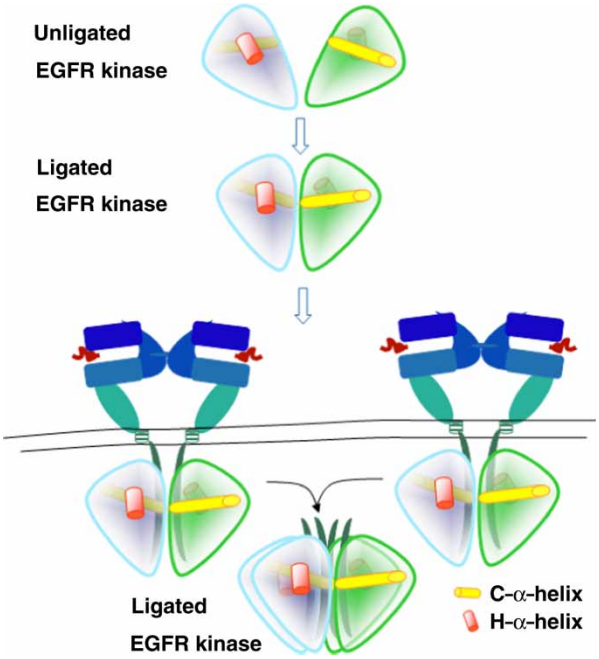

Figure 8. Model of EGFR conformational states, aggregation and activation on the cell surface. This model builds on the concept that a significant proportion of the high-affinity EGFRs is present on the cell surface as inactive dimers. At the top of the figure, an unligated, kinase-inactive dimer is depicted (the C- and H- α-helices do not interact in this conformation). On ligand binding, the EGFR-ECD domains reorient to form the back-to-back conformation, in this configuration the EGFR dimer is predicted to be kinase-inactive (the C- and H- α-helices are still not juxtaposed). However, in the back-to-back conformation, the ligated EGFR dimers oligomerize (e.g. to tetramers) juxtaposing the C- and H- α-helices from different dimers and activating the EGFR kinases. At the bottom of the figure, two views of the EGFR kinase-active tetramer are shown (side on and from the top). These cartoons of the EGFR-intracellular domains are based on the co-ordinates shown in more detail in Supplementary Figure 8.

the wtEGFR (i.e. $sEGFR_{1-621}$). Similarly, when the EGFR is expressed at low to normal levels (i.e. $<100{,}000$/cell) on the surface of Baf/3 cells, there is no binding of the mAb806 to the wtEGFR. Even when the wtEGFR is overexpressed on cell surface, antibody mAb806 (Johns et al. 2002; Garrett et al. 2009) recognizes $<10\%$ of the EGFR. Although the dynamics of binding of the EGFR- and erbB2-antibodies to cell surface EGFR and erbB2, respectively, require more detailed analyses (time of exposure and receptor density effects) (Walker et al. 2004), the allosteric effects of ligand binding are clear – the CR1 loop regions of both EGFR and erbB2 are less accessible to mAb806 and mAb3B10, respectively, after the EGFR binds ligand. On the cell surface, the mAb806 epitope on the wtEGFR is more exposed when erbB2 is also expressed. Previous results (Gadella and Jovin 1995; Clayton et al. 2005) suggest that a significant proportion of the EGFR must be present as pre-formed homodimers (Gadella and Jovin 1995; Clayton et al. 2005; Uyemura et al. 2005; Chung et al. 2010), or in the presence of erbB2 as EGFR:erbB2 heterodimers (Spivak-Kroizman et al. 1992; Karunagaran et al. 1996; Li et al. 2012). Although the tethered form of the wtEGFR (i.e. $sEGFR_{1-621}$) is very low affinity ($K_D \sim 400$ nm), it is not yet clear that the transition from the tethered to untethered conformation on the cell surface requires dimerization (homo- or hetero-) and/or interactions with intracellular modulators which may effect conformational (affinity) changes in the ECD (Bill et al. 2010). However, in the absence of ligand on the cell surface there will be a complex mixture of tethered, untethered, monomeric and dimeric (hetero and homo) EGFRs; some of these EGFR forms are also likely to be bound to cytoplasmic structures which may well alter their ligand affinity (Rijken et al. 1991; Walker and Burgess 1991). In all of these unligated receptor configurations, including the pre-formed dimers, the conformation of the EGFR-ECDs must constrain the orientation of the kinase domains to prevent the inappropriate activation of the kinase domain (Zhu et al. 2003). Measurements of YFP-EGFR expressed on the surface of living cells indicate that the ECD of a significant proportion of the pre-formed YFP-EGFR dimers are in an untethered conformation and the YFP is orientated away from the cell surface (i.e. the N-terminus is at least 8 nm from the membrane) (Kozer et al. 2011).

If the ECD domain of the EGFR is truncated, or ligand is present at a sufficient concentration, the conformation of intracellular domain changes, leading to activation of the receptor kinase. Truncation, such as the oncogenic truncation (Boerner et al. 2003; Garrett et al. 2009), prevents the ECD from appropriately regulating the configuration of the kinase domain and oligmer ization (either homo- or hetero-) leads to kinase activation (note ligand-independent activation signalling of Δ2-7EGFR/erbB2 in Figure 7). Presumably, ligand binding to the ECD shifts the conformations of the wtEGFR monomers and dimers towards the back-to-back dimer/oligomer conformation (Gadella and Jovin 1995; Ogiso et al. 2002; Garrett et al. 2003; Clayton et al. 2005) which is associated with a loss of mAb806 binding, increased oligomerization and receptor kinase activation. In the absence of reagents to quantitate the proportion of tethered receptors, it is difficult to measure the kinetics of all of the steps in the processes initiated by ligand binding and culminating in kinase activation. Similarly, the different forms of the higher affinity (untethered) EGFR-ECD (i.e. monomers, pre-formed dimers and/or EGFRs associated with cytoplasmic proteins) (Berkers et al. 1991; Rijken et al. 1991; Walker and Burgess 1991, 1988) are likely to be affinity modulated (Berkers et al. 1991; Bedrin et al. 1997), making the binding and activation kinetics difficult to interpret. There is increasing evidence that pre-formed, inactive oligomers are present on the cell

surface (Gadella and Jovin 1995; Uyemura et al. 2005; Clayton et al. 2007; Chung et al. 2010). In particular on Baf/3 cells the EGFR homodimers form tetramers on binding ligand (Clayton et al. 2005). Similarly, EGFR-kinase defective-erbB2 heterodimers (i.e. EGFR-V^{741}G-erbB2 or EGFR-K^{721}R-erbB) must form tetramers to phosphorylate (Spivak-Kroizman et al. 1992; Karunagaran et al. 1996) and activate the erbB2 kinase domain (see Figure 7 and Supplementary Figure 7). Consequently, we propose that in the ligand bound, back-to-back conformation the EGFR dimer forms higher order oligomers, e.g. tetramers, in such a way that the intracellular kinase domains are activated by an asymmetric interaction between the C- and the H- α-helices of different EGFR dimers (Figure 8, see also Supplementary Figure 8). Interestingly, the kinase domain structure (Walker et al. 1998b) and conformation (Gan et al. 2007) can also modulate the affinity of the EGFR-ECD for ligand and the mAb806 antibody. Inside-out signalling influences the biological sensitivity of the EGFR (Walker and Burgess 1988; Walker and Burgess 1991; Walker et al. 1993; deBlaquiere et al. 1994). A model for the potential complexes and activation states for membrane associated EGFR monomers, dimers and oligomers is presented in Supplementary Figure 6.

Progression of our understanding of the mechanism of activation of the EGFR kinases, from the ligand-dependent EGFR encoded tyrosine kinase monomer (Cohen et al. 1980; Ushiro and Cohen 1980), to the model that ligand induces receptor dimerization and consequential activation of the kinase dimer (Yarden and Schlessinger 1987), to models proposing that asymmetric activation of EGFR kinase dimers (Groenen et al. 1997; Zhang et al. 2006), to our current proposal: that ligand induces a conformational change in a pre-formed, untethered, kinase-inactive EGFR dimer, leading to a back-to-back configuration which aggregates to form an asymmetric, kinase-active EGFR tetramer (or higher order oligomers), has evolved from an extraordinary level of research (Schlessinger 2002). Analysis of the relationship between ligand binding and kinase activation of EGFR family members is confounded by changes in linkage (i.e. aggregation state), conformation (of both the extracellular and intracellular domains) and inside-out signalling (e.g. changes to the EGFR juxtamembrane domain, the kinase domain or the C-terminus). All of these changes will perturb the kinetics of ligand binding. Many models of receptor activation focus on a ligand-induced monomer-dimer transition (Dawson et al. 2005; Macdonald and Pike 2008; Macdonald-Obermann and Pike 2009; Liu et al. 2012; Macdonald-Obermann et al. 2012; Pike 2012), yet many biophysical studies demonstrate the presence of pre-formed homo- and heterodimers (Gadella and Jovin 1995; Uyemura et al. 2005; Clayton et al. 2008; Chung et al. 2010; Kozer et al. 2011). In the absence of ligand these higher order EGFR family oligomers appear to have little or no kinase activity. (N.B. When there are truncations to the EGFR-ECD, spontaneous activation of the EGFR kinase can be detected. It should also be noted that the constitutive level of intracellular tyrosine phosphatase activity is high (Mattoon et al. 2004), so low levels of endogenous EGFR kinase activity can be difficult to detect). The analysis of ligand-binding kinetics can also be confounded by the presence of low levels of autocrine ligand production. However, it is clear that the composition and density of EGFR family members on the cell surface effects the kinetics of ligand binding (Wilkinson and Staros 2002; Pike 2012). Quantitative models which focus on the monomer-dimer transition without considering the effects of higher order oligomers (pre-formed or ligand induced) are required. From our experiments, the effects of ligand binding to the EGFR on the accessibility of the erbB2-CR1 domain and the reciprocal effect of erbB2 on the accessibility of the EGFR-CR1 domain are clear.

The ligand induction of two different conformations of the EGFR intracellular domain, leading to kinase activation as proposed by Zhang et al. (2006) suggests that the ligand-bound dimer will be active. The dimer to tetramer kinase activation process proposed in this report predicts inactive EGFR dimers and kinase activation when higher order oligomerization occurs. In other receptor kinase activation systems (e.g. ephrin:eph interactions) higher order, hetero-oligomerization appears to dominate the kinase activation processes (Janes et al. 2011), it would not be surprising if similar higher order oligomerization activation processes occurred with of overexpressed (Kawamoto et al. 1983; Gadella and Jovin 1995), ECD truncated (Mishima et al. 2001) or clusters of ligand-bound EGFR family members (Gadella and Jovin 1995; Clayton et al. 2005, 2008). It is important that the 'simplest' cell surface experimental systems (i.e. minimal internalization, one EGFR family member at medium to low density and minimal autocrine ligand stimulation) are used to identify the relationship between the receptor aggregation state, ligand-binding kinetics and kinase activation. It will be interesting to continue developing real-time techniques (Chung et al. 2010; Kozer et al. 2011; Macdonald-Obermann et al. 2012) to probe the details of ligand-induced changes (Burgess et al. 2003; Garrett et al. 2009; Liu et al. 2012) on the structural dynamics of both the ECD and kinase domains (Shih et al. 2011) of EGFR family members on the membranes of both normal and tumour cells. It will also be helpful to produce probes which can quantitate the proportion of tethered EGFR on the cell surface and eventually to determine whether the EGFR dimer can have kinase activity or whether a higher order oligomer (e.g. a tetramer) is required for kinase activation.

Supporting information available

Supporting information includes eight figures with appropriate experimental procedures, and Supplemental References.

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Note

*These two authors contributed equally to this work.

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