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Negative co-operativity in the EGF receptor

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Abstract

Scatchard analyses of the binding of EGF (epidermal growth factor) to its receptor (EGFR) yield concave-up Scatchard plots, indicative of some type of heterogenity in ligand-binding affinity. This was typically interpreted as being due to the presence of two independent binding sites: one of high affinity representing $\leqslant$10% of the receptor population, and one of low affinity making up the bulk of the receptors. However, the concept of two independent binding sites is difficult to reconcile with the X-ray structures of the dimerized EGFR that show symmetrical binding of the two ligands. A new approach to the analysis of ^{125}I-EGF-binding data combined with the structure of the singly-occupied *Drosophila* EGFR have now shown that this heterogeneity is due to the presence of negative co-operativity in the EGFR. Concerns that negative co-operativity precludes ligand-induced dimerization of the EGFR confuse the concepts of linkage and co-operativity. Linkage refers to the effect of ligand on the assembly of dimers, whereas co-operativity refers to the effect of ligand binding to one subunit on ligand binding to the other subunit within a preassembled dimer. Binding of EGF to its receptor is positively linked with dimer assembly, but shows negative co-operativity within the dimer.

Early studies of the EGFR (epidermal growth factor receptor)

EGF (epidermal growth factor) was first purified from extracts of mouse submaxillary glands by Stanley Cohen nearly half a century ago, on the basis of its ability to promote precocious eyelid opening and accelerate incisor eruption in newborn mice [1]. A decade later, receptors for EGF were identified by radioligand-binding assay [2,3]. Although the earliest work showed linear Scatchard plots [2,3], it very quickly became apparent that the Scatchard plots for the binding of EGF to its cell-surface receptor (EGFR) exhibited upward concavity [4–6], indicating some type of heterogeneity in ligand-binding affinity.

There are two possible interpretations for a concave-up Scatchard plot. The first is that the system exhibits negative co-operativity, i.e. binding of ligand to the first site in a dimer reduces the affinity of the second site for the ligand. The second explanation is that there are two independent binding sites of differing affinity. By definition, in a dimer showing negative co-operativity, half of the sites should be high-affinity and half of the sites should be low-affinity. However, in the case of the EGFR, only $\sim$1–10% of the sites were high-affinity sites ($K_d \sim 50$ pM). The vast majority of the sites were low-affinity sites ($K_d \sim 3$ nM). Thus the experimental data did not align with the predictions for negative co-operativity and the interpretation of curvilinear Scatchard plots as being due to the presence of 'two independent classes of sites' became entrenched.

Key words: epidermal growth factor (EGF), epidermal growth factor receptor (EGFR), negative co-operativity, saturation binding isotherm.

Abbreviations used: CHO, Chinese-hamster ovary; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; dEGFR, *Drosophila* EGFR.

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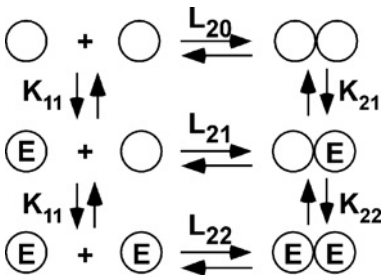
Characterization of the EGFR protein proceeded apace and, in 1980, Stanley Cohen's group at Vanderbilt reported that the EGFR co-purified with a protein kinase activity [7] that was subsequently shown to be a tyrosine kinase [8,9]. The receptor was cloned and sequenced in 1984 [10] and found to consist of a ligand-binding extracellular domain, a single-pass transmembrane domain and an intracellular kinase domain. In 1987, Yarden and Schlessinger [11] showed that the EGFR existed in membranes as a monomer, but dimerized upon addition of EGF. This resulted in the suggestion that the EGFR dimer represented the high-affinity binding site, whereas the monomer represented the low-affinity site. However, since EGF induced its receptor to form dimers, there should have been positive co-operativity in this system, namely a concave-down Scatchard plot, rather than negative co-operativity. Thus it was clear that this simple model was inadequate for explaining the binding properties of the EGFR.

The first crystal structures of the doubly liganded dimerized extracellular domain appeared in 2002 [12,13]. In 2003, the crystal structure of the effectively unliganded EGFR monomer was reported [14]. Together, they provide a clear picture of the effect of EGF binding on receptor dimerization. The extracellular domain of the EGFR comprises four subdomains, designated I–IV. In its monomeric form, the EGFR exists in a bent configuration, held together by intramolecular interactions between the so-called dimerization arm of subdomain II and the tethering arm of subdomain IV [14]. Upon binding EGF, this intramolecular tether is released and the receptor adopts an open configuration in which the dimerization arm is available to interact with the dimerization arm of a second receptor monomer. A back-to-back dimer is formed, mediated by receptor–receptor interactions [12,13].

Figure 1 | General model for the binding of ligand in a dimerizing system

Open circles represent unoccupied EGFR monomers. E represents EGF. A circle containing an E represents a ligand-occupied EGFR. See the text for further discussion.



Models of negative co-operativity

On the basis of these structures, it was proposed that EGF bound with high affinity to the open or extended form of the receptor, but with low affinity to the tethered form of the receptor. However, studies of the binding of EGF to receptor mutants [15] indicated that the extended form of the receptor did not represent the high-affinity EGF-binding site. Furthermore, mathematical modelling of the binding of EGF in a system in which a low-affinity tethered form of the receptor was in equilibrium with a high-affinity extended form of the receptor could not recapitulate the *in vivo* binding data [16]. Instead, an unspecified 'external' binding site that bound the receptor dimers with high affinity was invoked to fit the experimental data [16].

The reason that these modelling studies could not reproduce the binding properties of the EGFR was because the constraints applied to the model explicitly excluded negativity co-operativity [16]. Specifically, the affinity of EGF for binding to the second site on the dimer was constrained to be equal to the affinity of EGF for binding to the first site on the dimer. In fact, a decade earlier, Wofsy et al. [17] had used the same model, but without these constraints, and showed that EGF-binding data could theoretically be explained by the presence of negative co-operativity within the EGFR dimer. However, these workers did not provide experimental support for the larger predictions from this model.

Negative co-operativity in a dimerizing system

In the general model for the binding of EGF in a dimerizing system (Figure 1), the unoccupied EGFR monomer is in equilibrium with the unoccupied EGFR dimer. This reaction is described by the association constant, L_{20}. EGF can then bind to three different species: (i) the monomer; (ii) the first site on the dimer; and (iii) the second site on the dimer. The binding of EGF to the monomer is described by the association constant K_{11}. The binding of EGF to the first site on the dimer is described by the association constant K_{21}. And the binding of EGF to the second site on the dimer is described by the association constant K_{22}. As L_{21} and L_{22} are defined on the basis of microscopic equilibrium, only four parameters are needed to fully describe this model. Heterogeneity in ligand-binding affinities derives from differences in K_{11}, K_{21} and K_{22}.

For this model, the equation that describes the fractional saturation of the EGFR at any given concentration of EGF is:

$$\bar{Y} = \frac{K_{11}[\mathrm{EGF}] + L_{20}[\mathrm{R}]K_{21}[\mathrm{EGF}](1 + 2K_{22}[\mathrm{EGF}])}{1 + K_{11}[\mathrm{EGF}] + 2L_{20}[\mathrm{R}]\{1+K_{21}[\mathrm{EGF}](1 + K_{22}[\mathrm{EGF}])\}}$$

where [R] is the concentration of unoccupied EGFR monomers [18]. In turn, the concentration of unoccupied EGFR monomers depends on the total concentration of EGFRs in the cell, which can be calculated according to the equation derived by Wyman and Gill [19]:

$$R_0 = [\mathrm{R}](1+K_{11}[\mathrm{EGF}]) + 2L_{20}[\mathrm{R}]^2(1+K_{21}[\mathrm{EGF}] + K_{21}K_{22}[\mathrm{EGF}]^2)$$

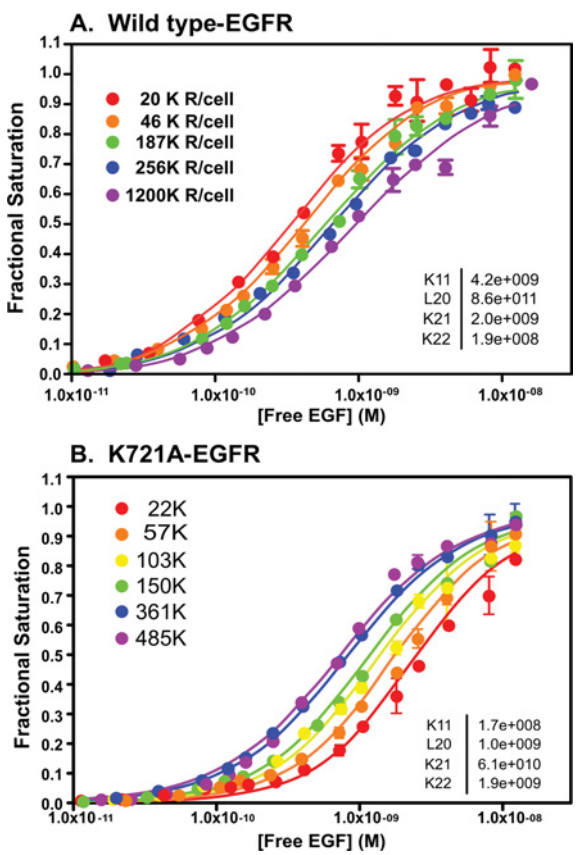
If the affinity of EGF differs for the monomeric and dimeric forms of the receptor, this model predicts that the saturation binding isotherm for EGF will shift position depending on the total number of EGFRs present in the cell. This is a simple consequence of mass action as more dimeric species will be present at high concentrations of receptor, whereas more monomeric species will be present at low concentrations of receptor.

In 2008, we tested this prediction by putting the EGFR on a tetracycline-inducible promoter and stably transfecting it into CHO (Chinese-hamster ovary) cells, which lack endogenous EGFRs. We then performed ^{125}I-EGF-binding studies on cells induced to express increasing levels of EGFRs [20]. Figure 2(A) shows an example of the data obtained in this type of experiment. The saturation binding isotherms shift from left to right with increasing numbers of EGFRs per cell. Global modelling of the data from the all the curves yields a single set of fitted values for all four equilibrium constants, K_{11}, K_{21}, K_{22} and L_{20}. The most striking finding from this analysis is that the affinity of EGF for the second site on the dimer (K_{22}) is substantially less than the affinity of EGF for the first site on the dimer (K_{21}). These data indicate that the general model for ligand binding in a dimerizing system is a valid description of EGF binding and demonstrate unequivocally that the binding of EGF to its receptor is negatively co-operative.

The existence of negative co-operativity in the EGFR would seem to be at odds with the demonstrated ability of the growth factor to induce the formation of receptor dimers. However, this logic is flawed because it fails to distinguish between the concepts of co-operativity and linkage. Co-operativity relates to the effect of the binding of ligand to one subunit in a preassembled dimer on the binding of ligand to the second subunit in that dimer. In contrast, linkage refers to the ability of a ligand to induce the assembly of dimers. Biochemical experiments indicate that the binding

Figure 2 | ^{125}I-EGF binding to CHO cells expressing wild-type or kinase-dead EGFRs

(**A**) CHO cells were stably transfected with a plasmid expressing wild-type EGFR from a tetracycline-inducible promoter. Cells were cultured for 48 h with increasing concentrations of doxycycline to induce receptor expression. ^{125}I-EGF binding was then carried out by incubating cells overnight at 4°C with 40 pM ^{125}I-EGF and increasing concentrations of unlabelled EGF. $e + x = \times 10^{x}$. (**B**) As (**A**) except that CHO cells were expressing the kinase-dead K721A-EGFR from the tetracycline-inducible promoter. This research was originally published in the *Journal of Biological Chemistry* [21]. (Macdonald-Obermann, J.L. and Pike, L.J., The intracellular juxtamembrane domain of the epidermal growth factor receptor is responsible for the allosteric regulation of EGF binding. J. Biol. Chem. 2009; 284:13570–3576.) © the American Society for Biochemistry and Molecular Biology.



of EGF to its receptor induces dimer formation, i.e. the EGFR exhibits positive linkage. This phenomenon is distinct from the negative co-operativity between subunits once the dimer is formed.

Positive linkage occurs when the affinity of a ligand for binding to the first site on the dimer is greater than that for binding to the monomer ($K_{21} > K_{11}$). The values of K_{21} and K_{11} shown in the inset of Figure 2(A) are nearly identical, suggesting that there is no linkage. However, this apparent absence of linkage is due to the fact that it is masked in the wild-type EGRF by the phosphorylation of the receptor that occurs subsequent to EGF binding. When the binding experiments are repeated in the kinase-dead K721A-EGFR (Figure 2B), the positive linkage become apparent [21]. In contrast with the situation in the wild-type receptor, the saturation binding isotherms for the kinase-dead EGFR shift from right to left with increasing numbers of receptors per cell. Global fitting of the data reveals that negative co-operativity is still present ($K_{21} > K_{22}$), but, in addition, positive linkage is present as K_{21} is nearly two orders of magnitude greater than K_{11}. Thus autophosphorylation of the receptor alters its ligand-binding properties, masking the positive linkage that is present in the native unphosphorylated receptor.

The obvious differences between the binding characteristics of the wild-type and kinase-dead EGFRs underscore the problem of relying on Scatchard plots for assessing the ligand-binding properties of the EGFR. Both wild-type and kinase-dead EGFRs yield concave-up Scatchard plots, suggesting that the loss of kinase activity does not change the way EGF binds to its receptor. However, analysis of data from multiple saturation binding isotherms reveals that the binding properties of these two receptors are actually quite different. A second problem with the use of Scatchard plots arises when curvilinear plots are broken down into two 'independent' sites, i.e. the high-affinity and low-affinity sites. In this situation, the values obtained for the number and affinity of the two classes of sites will vary depending on how many receptors are present in the cell. Thus the data cannot be used to support claims for differences in the fraction of 'high-affinity sites' between two receptor mutants as the values obtained, even using the same receptor, will vary depending on receptor number. Finally, the underlying assumption in the derivation of the binding equations of which the Scatchard equation is a transformation, is that only a small fraction of the input ligand is bound. If the ligand-binding assays are done under conditions in which more than ~10% of the input ligand is bound, the Scatchard equation cannot be used to analyse the binding data [22]. Scatchard plots of such data show downward concavity, which is an artefact of the 'over-binding' of ligand.

Structural basis for negative co-operativity

By performing binding analyses on cells expressing increasing levels of various mutant forms of the EGFR, it is possible to obtain information on the structural requirements of the receptor for supporting negative co-operativity. Subdomain IV of the extracellular domain contains the tethering arm that is thought to interact with the dimerization arm in subdomain II to hold the EGFR monomer in its closed conformation [14]. Mutation of the three residues thought to be involved in this tethering interaction, Asp^{563}, His^{566} and Lys^{585}, does not significantly alter the ligand-binding properties of the receptor [23]. This suggests that the tethering interactions are not involved in the allosteric regulation of ligand binding.

The tethering arm in subdomain IV contains two disulfide loops: (i) Cys^{558}–Cys^{567}; and (ii) Cys^{571}–Cys^{593}. Deletion of

the entire Cys^{558}–Cys^{567} loop had only a modest effect on co-operativity and linkage in the EGFR. Similarly, release of this disulfide by substitution of alanine residues for the cysteine residues also had little effect on allostery in ligand binding. In contrast, deletion of the entire Cys^{571}–Cys^{593} loop abrogated linkage and co-operativity in ligand binding. Release of this loop by double alanine substitution resulted in enhanced negative co-operativity [23]. These findings link the extracellular juxtamembrane domain, and particularly the region between Cys^{571} and Cys^{593} to negative co-operativity.

Nested truncations from the C-terminus of the EGFR have also implicated the intracellular juxtamembrane domain in negative co-operativity [21]. Truncation of the C-terminal tail of the receptor (c'973-EGFR) yielded values for the four equilibrium association constants that were similar to those for the kinase-dead receptor and indicated the presence of both positive linkage and negative co-operativity. Surprisingly, deletion of the kinase domain as well as the C-terminal tail of the EGFR (c'698-EGFR) also yielded a receptor with intact positive linkage and negative co-operativity. In contrast, deletion of the entire intracellular domain led to the production of a receptor that showed neither co-operativity nor linkage. These findings suggest that the intracellular juxtamembrane domain of the EGFR is structurally involved in the genesis of negative co-operativity. Consistent with this interpretation is the finding that replacing residues 647 and 650, just beyond the membrane, with cysteine residues, which enables palmitoylation of the receptor, also led to the complete loss of negative co-operativity [21]. Thus it appears that the membrane-proximal portion of both the extracellular and intracellular domains of the EGFR are involved in generating negative co-operativity.

Recently, Alvarado et al. [24] reported an X-ray crystal structure of the *Drosophila* EGFR (dEGFR), which yielded important insights into the structural basis for negative co-operativity in that receptor system. In contrast with the human EGFR, which does not retain negative co-operativity when purified and assayed as the soluble extracellular domain, the isolated extracellular domain of the dEGFR does show negative co-operativity [24]. The crystal structure of the singly liganded dimer of the dEGFR shows an asymmetric dimer in which the second, unoccupied, site is conformationally restrained and thus would have a significantly lower affinity for ligand than does the first site. Given the homologies between the human EGFR and dEGFR, it seems likely that the human EGFR also forms an asymmetric singly ligated extracellular domain dimer with an important difference being that the intracellular juxtamembrane domain of the human EGFR also contributes to this asymmetry.

Conclusions

Novel analyses of ligand-binding data have demonstrated that the observed heterogeneity of EGF-binding affinity results from negative co-operativity [20,21,23]. High-affinity binding occurs to the first site on the receptor dimer, whereas low-affinity binding occurs to the second site on the dimer as well as to the monomer. The existence of negative co-operativity in the EGFR is supported further by the X-ray crystallographic structure of dEGFR [24]. Additional studies are needed to explicitly define the structural basis of negative co-operativity in the mammalian EGFR and to determine how it contributes to the regulation of cell signalling.

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