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Ligand-induced ErbB receptor dimerization

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Abstract

Structural studies have provided important new insights into how ligand binding promotes homodimerization and activation of the EGF receptor and the other members of the ErbB family or receptor tyrosine kinases. These structures have also suggested possible explanations for the unique properties of ErbB2, which has no known ligand and can cause cell transformation (and tumorigenesis) by simple overexpression. In parallel with these advances, studies of the EGF receptor at the cell surface increasingly argue that the structural studies are missing key mechanistic components. This is particularly evident in the structural prediction that EGF binding linked to receptor dimerization should be positively cooperative, whereas cell-surface EGF-binding studies suggest negative cooperativity. In this review, I summarize studies of ErbB receptor extracellular regions in solution and of intact receptors at the cell surface, and attempt to reconcile the differences suggested by the two approaches. By combining results obtained with receptor ‘parts’, it is qualitatively possible to explain some models for the properties of the whole receptor. These considerations underline the need to consider the intact ErbB receptors as intact allosterically regulated enzymes, and to combine cellular and structural studies into a complete picture.

Keywords

Epidermal growth factor receptor; Receptor tyrosine kinase; ErbB receptor; Cooperativity; Receptor dimerization; Crystal structure; Heterodimer; Autoinhibition; Ligand binding; Allostery

INTRODUCTION

The epidermal growth factor receptor (EGFR) is one of the most well-studied receptor tyrosine kinases (RTKs), and one of the first for which ligand-induced dimerization was proposed as a primary event in transmembrane signaling [1–4]. In mammals, EGFR is one of a family of four RTKs, collectively known as the ErbB receptors [5] that includes ErbB2/HER2/Neu [6] plus the neuregulin receptors ErbB3/HER3 [6] and ErbB4/HER4 [7]. Each has a large extracellular ligand-binding region of ~620 amino acids, a single membrane-spanning α -helix, and a ~550 amino acid intracellular region that contains a juxtamembrane region (~45aa) followed by a tyrosine kinase domain (~270aa) and carboxy-terminal regulatory sequences (~230aa).

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It is now well accepted that binding of EGF (or other agonists) to EGFR shifts a monomer $\rightleftharpoons$ dimer equilibrium to favor the dimeric state [8]. Similarly, neuregulin binding to ErbB4 promotes homodimerization of this receptor [7]. Although the mechanism is currently far from clear [9], it is also thought that the four ErbB receptors form an array of heterodimers upon ligand binding [10], potentially thus increasing the complexity of signaling by this family. Enhanced (or altered) receptor homodimerization or heterodimerization controls activation of the intracellular tyrosine kinase domain. In EGFR and ErbB4 homodimers, this occurs through an allosteric mechanism reviewed elsewhere in this issue by Bose and Zhang [11]. Kinase activation leads to ErbB receptor autophosphorylation, which promotes the recruitment of downstream signaling proteins that contain Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains [12] and consequent modulation of a complex signaling network [13].

A major breakthrough in understanding how ErbB receptors are regulated by their growth factor ligands coincided with the publication of the last ErbB/EGF Mini Review Issue of *Experimental Cell Research* in 2003. In 2002 and 2003, X-ray crystal structures were published of the complete extracellular regions of EGFR [14], ErbB2 [15–17] and ErbB3 [18] without activating ligand. In addition, structures of a large part of the EGFR extracellular domain in ligand-induced dimers were described [19,20]. These structures, together with an unliganded ErbB4 extracellular structure published in 2005 [21], laid the foundation for a satisfying structural model of ligand-induced ErbB receptor dimerization [22] that is summarized in Figure 1. Together with other studies, these advances also suggested explanations for the known differences between members of the ErbB receptor family [22,23]. In the past 5 years, it has become clear that the mechanism of ligand-induced ErbB receptor activation is more complicated than suggested in Figure 1. In addition, it was always clear that the structure-based models for ligand-induced dimerization cannot adequately explain the characteristic features of EGF binding to EGFR at the cell surface [22]. In this Minireview, I shall discuss advances in our understanding of ErbB receptor activation in the past few years, focusing in particular on key results that provide insight into mechanisms of ligand-induced receptor activation at the cell surface. Taken together, the recent advances argue that considering the intact ErbB receptors as complete allosteric enzymes – as indeed was suggested some 20 years ago [24] – will be crucial for a full understanding of ErbB receptor activation mechanisms.

Mechanism of ligand-induced EGFR dimerization from structures of extracellular regions

Figure 1 presents the essential features of the 2003 structure-based model [22] for EGF-induced dimerization of the EGFR extracellular region. Mammalian ErbB receptor extracellular regions contain four distinct domains. Domains I and III (red in Figure 1) are both β -helix solenoid structures, and share 37% sequence identity in EGFR. These domains are related to the leucine-rich repeat superfamily [25], and are responsible for ligand binding – with both domains simultaneously contacting the same bound ligand. Domains II and IV (green in Figure 1) are both cysteine-rich domains with disulfide bond connectivities similar to those seen in laminin and the tumor necrosis factor receptor [26]. The insulin and insulin-like growth factor receptors share both types of domain with the ErbB receptors [27].

In a dimer of EGFR extracellular regions, all intermolecular contacts are mediated by the receptor [19,20], making EGFR unique among RTKs with known ligand-bound structures. In all other such cases, the ligand contributes directly to the dimer interface [28,29]. Dimerization of the EGFR extracellular region (sEGFR) is largely mediated by its ‘dimerization arm’ [20], which projects from the cysteine-rich domain II (marked in Figure 1). In addition, parts of domain IV are thought to come close (or into direct contact) at the dimer interface based on both biochemical studies [30] and modeled structures [14]. Direct domain IV interactions could not be visualized crystallographically in the dimer of the complete EGFR extracellular region [20]. However, recent negative stain electron microscopy studies of intact EGFR [31] and

small-angle X-ray scattering (SAXS) of the isolated extracellular region [32] indicate that the modeled location of domain IV in the right-hand part of Figure 1 is correct.

In the absence of bound ligand, the EGFR extracellular region adopts a very different ‘tethered’ configuration, in which the domain II dimerization arm is buried by intramolecular interactions with domain IV [14,18,22]. As illustrated in Figure 1, the ligand-free ‘tethered’ configuration and the ligand-bound ‘extended’ configuration are mutually exclusive. When ligand binds, it contacts sites on both domains I and III and draws these two domains together. The resulting conformational change ‘extends’ the receptor, and exposes the dimerization interfaces in domain II and domain IV for productive interactions with another ligand-bound receptor molecule. Thus, ligand binding to EGFR promotes a dramatic conformational change that is required to break domain II/IV interactions in the tethered configuration that autoinhibit dimerization. It has been generally assumed that, by burying the dimerization arm, the existence of the tethered conformation reduces receptor dimerization in the absence of ligand [33].

Structures of other ErbB receptor extracellular regions

The model presented in Figure 1 also provided a satisfying framework for understanding the properties of the other human ErbB receptors based on the structures of their extracellular regions. As shown in Figure 2, unliganded extracellular regions of ErbB3 [18] and ErbB4 [21] also adopt the characteristic tethered configuration, suggesting that these receptors are similarly autoinhibited. Neuregulin binding to these receptors is thought to promote an extension of the molecule (and exposure of the dimerization arm) similar to that seen when EGF or TGF α bind to EGFR, and this has been confirmed by X-ray scattering studies of the ErbB3 extracellular region in solution [32]. By stark contrast, structures of the ErbB2 extracellular region [15–17] showed it to be extended even in the absence of bound ligand. ErbB2 has no known soluble activating ligand [6], and is unique among the ErbB receptors in being able to transform cells by simple overexpression [34]. ErbB2 overexpression is also strongly associated with breast cancer [35] –and it is the target of the Herceptin antibody [15, 36]. These facts suggested that ErbB2 is a ‘ligandless’ receptor [6] that is constitutively ‘poised’ by virtue of its exposed dimerization arm either to homodimerize constitutively or to heterodimerize with other ErbB receptors following their binding to cognate ligands [15,17].

Although these structures of ErbB receptor extracellular regions provided some satisfying explanations for their unique properties, numerous key questions about how ErbB receptor homo and heterodimerization is specified and driven remain unanswered. At the level of the isolated extracellular regions, biophysical studies have shown clearly that ligand binding can promote strong homodimerization of EGFR and ErbB4 only [9]. Neuregulin binding to the ErbB3 extracellular region does not promote its dimerization [9,32,37,38]. In fact, some reports suggest that the ErbB3 extracellular region oligomerizes weakly in the *absence* of ligand [18, 37], and that ligand binding actually reverses this [37]. Moreover, it is important to appreciate that the ErbB2 extracellular region has shown no sign of ligand-independent homodimerization either in solution biophysical studies [9,38] or in crystals [15,17]. It is tempting to suggest that these results with ErbB2 and ErbB3 simply illustrate shortcomings of studying the extracellular regions in isolation. However, studies with full-length chimeric receptors argue that neuregulin does not promote ErbB3 homodimerization in the plasma membrane of cells lacking other ErbB receptors [39]. Moreover, quantitative analyses using an enzyme complementation approach at the cell surface have shown that ErbB2 homodimerizes no more strongly than EGFR in the absence of ligand [40]. Thus, quantitative studies argue that robust homodimerization is restricted to EGFR and ErbB4. Neither ErbB2 nor ErbB3 homodimerizes. It is true that some qualitative crosslinking and activation studies have indicated ErbB2 homodimerization, but these reports have typically employed either very high-level transient overexpression of ErbB2 [41] or cancer cells that have an amplified ErbB2 gene (leading to

the expression of $>10^6$ ErbB2 molecules per cell) and also express other ErbB receptors (and frequently ErbB ligands). ErbB2 dimerization seen under these circumstances appears to be no more extensive than the so-called pre-formed dimers reported for EGFR in the absence of ligand (see below).

ErbB receptor heterodimerization

Whereas the homodimerization properties of ErbB receptors appear to be faithfully recapitulated by solution and crystal studies of their isolated extracellular regions, this is not true for heterodimeric interactions. In our own solution biophysical studies, we observed weak heterodimerization of ErbB2 with the neuregulin-bound extracellular regions of ErbB3 or ErbB4 [9], but failed to detect EGF-induced association of the EGFR and ErbB2 extracellular regions (or other anticipated heterodimers). Horan *et al.* [38] could not detect ErbB2/ErbB3 heterodimers in similar experiments. By contrast, several studies at the cell surface have demonstrated – with intact receptors or various chimerae – robust association of ErbB2 with EGFR [40] and ErbB3 [39,40,42] in a cellular context, in response to EGF and neuregulin respectively. Studies of full-length receptor phosphorylation [43] and of interactions between chimeric receptors with an intracellular reporter domain [40] are also consistent with a model in which ErbB2 forms hetero-oligomers with ligand-bound EGFR, ErbB3 or ErbB4. Quantitative studies at the cell surface [40], and conclusions from modeling analyses [44], argue that EGFR/ErbB2 heterodimers form with a similar (and *not* greater) affinity to EGFR homodimers. ErbB3/ErbB2 heterodimers appear particularly robust, in part presumably because the competing homodimerization reactions are very weak. In fact, these two receptors rely substantially on heteromeric interactions with one another for signaling and cell transformation [45]. Heterodimers involving EGFR with ErbB3 or 4 appear to be significantly weaker [40,43], if they form at all.

The fact that isolated extracellular regions recapitulate homodimeric ErbB receptor interactions better than heterodimerization suggests that other regions of the receptors may play a crucial role in associations that involve two different receptors. For example, the transmembrane domains of ErbB receptors have been reported to self-associate in membranes, through a well-characterized GxxxG-like motif [46]. Interestingly, recent FRET studies of synthetic peptides in detergent solution [47] have suggested that hetero-oligomerization of the ErbB2 transmembrane domain with the corresponding region of EGFR or ErbB3 is substantially favored over homomeric interactions involving ErbB receptor transmembrane helices (or other heteromers). Earlier studies [48,49] have shown that mutating the GxxxG-like dimerization motif in the EGFR transmembrane domain does not prevent normal signaling by this receptor (through homodimerization). However, no reported studies have addressed the importance of transmembrane domain dimerization motifs in ErbB receptor heterodimerization. ErbB receptor intracellular domains also self-associate in defined ways [11,50], and these interactions could contribute directly to ErbB receptor dimerization/oligomerization, and possibly to defining heterodimerization specificity.

Role of the autoinhibitory tether in ErbB receptor regulation

Mutations [14] or deletions [51] that break the domain II/IV tether in the isolated EGFR extracellular region were shown to increase its affinity for EGF or TGF α by up to 30-fold. This reflects a reduction (of ~ 2 kcal/mol) in the energetic barrier to bringing domains I and III sufficiently close to contact simultaneously the same ligand molecule (see Figure 1). However, exposing the domain II dimerization arm with these mutations is not sufficient to promote sEGFR dimerization in the absence of ligand [51,52]. Even when almost all of domain IV is deleted, so that the tether is completely abolished (and domain II fully exposed), sEGFR dimerization cannot be detected in the absence of EGF or TGF α [19,51]. This finding argues that ligand binding must do more than simply expose the dimerization arm in order to promote

receptor-receptor interactions. Indeed, regions outside the dimerization arm also contribute to EGFR dimerization [19,20], as marked with asterisks in Figure 1 (top right). Q194 side-chains make contacts across the interface (upper asterisk), as do D279 and H280, immediately to the C-terminus of the dimerization arm (lower asterisk in Figs. 1 and 3). Alanine substitution at D279/H280 essentially abolishes EGFR dimerization [52], although Q194 substitution has relatively little effect.

As shown in Figure 3, domain II of sEGFR adopts significantly different conformations in the active (green [20]) and inactive (red [53]) structures. In Figure 3A, the domain I/II portion of each structure is shown, overlaid using domain I as a reference. Although the four N-terminal disulfide-bonded modules of domain II overlay well, the trajectory of domain II deviates substantially after module 4, so that the orientation of the dimerization arm and the remainder of domain II are quite different. Ligand binding appears to impose a precise ‘bend’ on domain II that may be required to allow the multiple contact sites marked by asterisks in Figure 3B to cooperate effectively with the dimerization arm in driving high-affinity dimerization [52]. Simply exposing the dimerization arm is not sufficient to control the receptor: ligand binding must instead remodel the entire dimerization interface by bending domain II in a precise way. Since the activating ligand interacts with both domains I and III, and these lie at either end of domain II, different ErbB ligands might promote distinct domain I/III relationships. In turn, these ligands might stabilize different degrees of domain II curvature, which could play a role in determining heterodimerization specificity.

Since abolishing the domain II/IV tether does not promote ligand-independent dimerization of sEGFR [19,51], it would not be predicted to cause constitutive EGFR activation in the full-length context. Indeed, studies of mutated intact EGFR seem to confirm this [54,55]. Mutations designed to disrupt the domain II/IV tether caused no apparent elevation in constitutive autophosphorylation of EGFR [54,55], although slight differences in dose-response relationships were seen for some EGF-dependent signals, consistent with increased ligand-binding affinity. Moreover, the rate of EGF dissociation from the cell surface was depressed by mutations predicted to disrupt the tether [54,56], in agreement with the increase in EGF binding affinity reported for analogous mutations in the isolated extracellular region. Similarly, differences were seen in the Scatchard plots obtained when studying ^{125}I -EGF binding to the cell surface [54–56] – although the discussion presented below makes it difficult to interpret this observation mechanistically. One thing that does seem quite clear is that the ‘high-affinity’ and ‘low-affinity’ sites discussed below do not simply reflect extended and tethered EGFR respectively.

It is important to take into account one additional recent biophysical observation when interpreting these studies. Using small angle X-ray scattering (SAXS) studies, Dawson *et al.* [32] found that mutations expected to disrupt the domain II/IV tether have a surprisingly small effect on sEGFR conformation. SAXS studies in solution readily distinguished tethered sEGFR from extended sErbB2 [32]. Moreover, a ligand-induced transition of sErbB3 from a tethered to extended configuration could be seen clearly. However, even when all crystallographically-observed intramolecular tether contacts had been abolished with mutations, sEGFR remained in a conformation that was indistinguishable at low resolution from tethered wild-type sEGFR. The mutations used in this work were more extensive than most of those employed in the cellular studies mentioned above – raising the possibility that the effects of EGFR tether disruption at the cell surface have not yet actually been properly tested.

The finding that disrupting all domain II/IV contacts in sEGFR does not yield an extended molecule also provides new insight into structural restraints in this molecule. In particular, Dawson *et al.* [32] pointed out that the linkage between domains II and III, which comprises the axis for the major conformational change (marked with a black circle in Figure 1), might

be more rigid than generally supposed. If this linkage has a preference for the configuration shown on the left-hand side of Figure 1, this would serve to stabilize the tethered form of EGFR. Interestingly, the equivalent linkage between domains C1 and L2 in the insulin and IGF1 receptors has a similar configuration [27,32]. Moreover, in genetic screens with *C. elegans*, alterations that disrupt the disulfide bond that appears to define the geometry of this linkage were identified as gain-of-function mutations in the EGFR orthologue LET-23 [57,58]. Extracellular, activating, EGFR mutations found in glioblastoma [59] are also not consistent with the simple hypothesis that the domain II/IV tether is key to autoinhibition of the receptor as suggested by Figure 1. More detailed study of these mutated forms of EGFR is likely to provide valuable insight into activation mechanisms.

Structural studies fail to explain ligand-binding characteristics at the cell surface

Perhaps the biggest disappointment of the ErbB receptor structural studies published in 2002–2003 is that they cannot explain the characteristic curvilinear (concave-up) Scatchard plots first reported for EGF binding to cell-surface receptors over 25 years ago [60,61]. Such binding characteristics signify either negative cooperativity or heterogeneity of sites, and have traditionally been interpreted to suggest the existence of two classes of EGF-binding site at the cell surface [61,62]. In this analysis, one ('high-affinity') class accounts for ~10% of receptors, with a K_D around 3×10^{-10} M. The remainder are 'low-affinity' receptors with a K_D of approximately 2×10^{-9} M. There has been much discussion of the nature of the 'high-affinity' and 'low-affinity' sites. Certain monoclonal antibodies have been reported to have preference for one or other class of sites [63,64]. Protein kinase C activation (through EGFR phosphorylation at T654) reduces the number of high-affinity EGF-binding sites at the cell surface [65], and deletions from the intracellular region of EGFR prevent the receptor from forming high-affinity sites [56,66].

Models in which the high-affinity sites represent EGFR dimers, and low-affinity sites represent monomers cannot explain the observed binding characteristics. All such models lead to positive cooperativity and thus concave-down (rather than concave-up) Scatchard plots [67]. Biophysical studies of the isolated extracellular region of human EGFR (sEGFR) suggested parameters for EGF-binding and EGFR dimerization consistent with positive cooperativity [68], which is also predicted by the structural model presented in Figure 1 [22]. Considering just two sEGFR molecules that do not interact without ligand: binding of the first EGF molecule will cause one sEGFR to become extended so that its dimerization arm is exposed. When the second sEGFR molecule binds EGF, its dimerization with the already-occupied sEGFR molecule will promote extension and thus increase the effective EGF-binding affinity. With these considerations, and using parameters based on solution studies of sEGFR, Klein *et al.* [56,69] could only account for the concave-up Scatchard plots seen at the cell surface by invoking an 'external site' that independently stabilizes a fraction of receptor molecules in a high-affinity dimeric state. Other studies have reached similar conclusions [70], and still others have argued that heterogeneities in receptor density can explain the observed Scatchard plot shape for EGF binding to cell-surface EGFR [71]. Wiley and colleagues pointed out that assuming such heterogeneities improved their ability to fit concave-up Scatchard plots [67], but only when they assumed negative, rather than positive, cooperativity in EGF binding to its intact receptor. A key requirement of their model is that EGFR dimers form with only one occupied receptor (R_2L , see Figure 4). Binding of a second EGF molecule, to form the doubly-occupied R_2L_2 dimer, must occur with lower affinity (by ~100-fold) than the first EGF-binding event – this constituting the suggested negative cooperativity. The preferential formation or R_2L dimers is also predicted by other analyses [72], although solution studies with the isolated human EGFR extracellular region have not provided any evidence for sEGFR₂/EGF formation [9,68,73,74].

Evidence for negative cooperativity in EGF binding to cell-surface EGFR

Recent studies from the Pike laboratory [75] provide significant insight into longstanding questions about the nature of EGF binding to cell-surface EGFR. Using an inducible expression system to control EGFR expression, Macdonald and Pike analyzed binding of ^{125}I EGF to pools of cells with six different (and known) levels of EGFR at their surfaces (from 24,000 to 500,000). Global fitting of the resulting data [75] yielded a best-fit model characterized by negative cooperativity of the sort predicted by Wiley and colleagues [67], summarized in Figure 4 (with details in the legend). Moreover, direct evidence for negative cooperativity was obtained with the demonstration that adding excess unlabeled EGF accelerates dissociation of labeled EGF from a high-affinity class of receptors at the cell surface [75,76]. A similar analysis with insulin provided the key piece of evidence for negative cooperativity in binding of insulin to its receptor [77,78].

A key requirement for negative cooperativity in EGF binding to the cell surface EGFR is that the singly occupied R_2L is the majority species at subsaturating EGF levels (Figure 4). The model also predicts that the receptor oligomerizes significantly in the absence of ligand. Studies with mutated receptors argued that the intracellular domain plays a significant role in promoting the formation of R_2L dimers [75]. This may explain why R_2L dimers are not seen in studies of the isolated EGFR extracellular region, and why the Scatchard plot curvature is absent in studies of EGF binding to EGFR lacking its intracellular domain [56,66] or to sEGFR. It is interesting to note that negative cooperativity in the insulin receptor is similarly lost when its extracellular region is liberated from the cell surface [77]. As with sEGFR, linear Scatchard plots reflecting only 'low affinity' insulin binding are obtained in studies of the soluble extracellular region of the insulin receptor. However, fusing a leucine zipper [79] or dimerizing immunoglobulin G fragments [80] to the carboxy terminus restores Scatchard plot curvature analogous to that seen with intact insulin receptor.

In the case of EGFR, the documented interactions between transmembrane domains [46,47] will contribute to receptor dimerization. In addition, several studies have shown that the intracellular domain of EGFR can oligomerize independently [11,50,81] in a manner that leads to its activation. Crystal structures of the isolated EGFR and ErbB4 tyrosine kinase domains [11,82,83] show precise asymmetric interactions that appear to be required for allosteric activation of the enzyme, as reviewed by Bose and Zhang [84]. It is possible that these asymmetric intracellular domain interactions indirectly impose asymmetry on the extracellular region of the EGFR receptor (and other ErbB receptors) that is required for manifestation of the negative cooperativity seen in ligand binding studies at the cell surface. One thing that emerges clearly from these considerations is that the properties of the whole receptor must be considered if we are to understand the details of its function. It is probably most fruitful to consider intact EGFR and other RTKs in the same way as other typical allosteric enzymes that undergo reversible oligomerization [24]. They are unique in being restricted to two dimensions, and in spanning the membrane. However, allosteric regulation of these enzymes by bound ligands results from precise alterations in domain-domain interactions in the context of oligomers of the whole enzyme.

Pre-formed EGFR dimers

In considering the EGFR as an allosteric enzyme as outlined above, it is to be expected that it may dimerize significantly in the absence of ligand [8]. Indeed, the insulin receptor is a covalently-linked pre-formed dimer that nonetheless requires insulin binding for activity [77]. Just as insulin stabilizes an active form of the insulin receptor dimer, so must EGF binding stabilize an active dimer of the EGF receptor. Indeed, early studies that showed signaling by insulin receptor/EGFR chimeras [85] argued that the regulatory mechanisms of these two receptor classes are closely related. In line with these expectations, a significant volume of

literature discusses evidence for the existence of EGFR dimers at the cell surface in the absence of EGF treatment [86–96]. The studies are based on a wide array of experimental techniques, and in many different cellular contexts (including A431 cells where autocrine EGFR activation is a problem [97]). Defining the nature of the dimers presumed to be in equilibrium with EGFR monomers at the cell surface is a central challenge in understanding EGFR regulation. For example, these ‘pre-formed’ dimers might represent the **R₂** species in Figure 4. Ligand binding might then stabilize the **R₂L** species and promote a conformational change within the dimer that also activates the intracellular kinase domain. Lower affinity binding of a second EGF in Figure 4 might then also contribute to dimer stabilization or alternatively promote dimer dissociation [75], depending upon the ligand.

Interestingly, a species interpreted as **R₂L** has been reported based on single molecule approaches to analyze binding of rhodamine-labeled EGF to HeLa cells [91]. However, contrary to the models outlined above, kinetic analysis in these studies was interpreted to imply positive cooperativity, with binding of a second ligand to the **R₂L** complex occurring approximately 10-fold more rapidly than binding of the first. One concern in these studies is that they follow only binding of fluorescently labeled EGF, and do not include any direct assessment of receptor oligomer size. Other studies suggest that the oligomers to which EGF is binding might exceed dimers [90,93], complicating interpretation. Moreover, Clayton *et al.* [90] and others [87,98] have observed fluorescence energy transfer (FRET) between EGF molecules at the cell surface, despite the fact that the two EGF molecules shown in the dimer in Figure 1 are more than 100Å apart, which is too distant for efficient FRET. This finding suggests FRET between dimers, in turn arguing that the single EGF-binding events observed by Teramura *et al.* [91] might represent binding to distinct dimers rather than reflecting positively cooperative binding to a single dimer.

Concluding Remarks

It is clear that a more thorough analysis of the nature of EGF-binding sites on the cell surface – or of the intact EGFR structure – is required for a full understanding of EGFR regulation. The structures of extracellular regions described here, and of intracellular regions reviewed by Bose and Zhang [84], have provided the essential tools for generating detailed hypotheses. The loss of negative cooperativity in EGF binding upon intracellular domain deletion [56,66] or isolation of the extracellular domain suggests that these manipulations remove crucial determinants of allosteric regulation. Similar experiences with the insulin receptor [77] further highlight this problem. In the past few years, understanding of the allosteric regulation of the EGFR kinase domain has advanced dramatically [11,84]. Progress is now being made in studies of the regulatory role played by the transmembrane, juxtamembrane and carboxy terminal regions of the receptor [47,50,99,100]. Each is likely to contribute directly to receptor dimerization or allosteric regulation. The challenge for the future – and it is a difficult challenge – is to move away from this fragmented approach to studying EGFR dimerization and activation, and to integrate detailed views of all parts of the receptor in an overall picture of how these allosteric enzymes are regulated.

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Figure 1.

Model for EGF-induced dimerization of the EGFR extracellular region. The top panel shows ribbon representations of sEGFR structures with- and without bound EGF. The left-hand structure is from Ferguson *et al.* [14], and shows the domain II/IV tether (ringed with orange oval) that occludes the dimerization arm. EGF binding to this structure induces a conformational change that can be modeled approximately by a 130° rotation of the domain I/II fragment about the axis between domains II and III marked by the black circle, in addition to a 20Å translation into the page. This change causes EGFR to adopt the extended conformation, in which the dimerization arm is exposed to drive dimerization as shown in the right-hand panel. The sEGFR dimer structure is based on the coordinates of the EGF/sEGFR complex published by Ogiso *et al.* [20]. We have modeled domain IV in this structure based on the domain III/IV relationship seen in unliganded sEGFR. Dimerization arm contacts at the dimer interface are ringed with an orange oval. Domains I and III are colored red and red/grey respectively. Domains II and IV are colored green and green/grey respectively. EGF is cyan. The lower panel shows a cartoon representation of this dimerization reaction, with each domain presented as a red or green rectangle.

Figure 2.

Structures of all four human ErbB receptor extracellular regions without bound ligand. EGFR, ErbB3 and ErbB4 all adopt the tethered conformation in the absence of ligand, whereas ErbB2 adopts a tethered conformation that resembles the ligand-activated, dimerization-competent, EGFR protomers in the EGFR dimer shown in Figure 1. Structures are shown in ribbon representation, with colors as described for Figure 1. The sEGFR structure is from Li *et al.* [53], sErbB2 is from Cho *et al.* [15], sErbB3 from Cho and Leahy [18], and sErbB4 from Bouyain *et al.* [21].

Figure 3.

Remodeling of the domain II dimerization interface of EGFR upon EGF binding. In panel **A**, the domain I/II fragments of sEGFR without bound ligand (1YY9 [53], shown in red) and with bound EGF (1IVO [20], shown in green) are overlaid with domain I as the reference. Although the first four disulfide-bonded modules of domain II overlay very well between the two structures, the trajectory of the domain is altered substantially beyond a point immediately N-terminal to the dimerization arm. As a result, the dimerization arm is reoriented substantially as shown by a double-headed black arrow. The C-terminus of domain II (where it links to domain III) is also repositioned significantly (also marked by double-headed curved arrow). As a result, the dimer contact site involving D279 and H280 (marked with an asterisk) lies in a very different position in the red inactive structure than in the green active structure. The different curvature imposed on domain II is thus proposed to 'remodel' the dimerization interface so that multiple weak contacts can cooperate with the dimerization arm in driving high affinity dimerization as described in the text. In **B**, one monomer from the EGF-bound sEGFR dimer [20] is shown. As mentioned in the legend to Figure 1, much of domain IV was missing from this structure. EGF is magenta, sEGFR is green. D279 and H280, which make crucial contacts across the dimerization interface [52], are marked. As discussed in the text, EGF (or another ligand) is thought to impose a precise bend on domain II (shown as a cyan curve against domain II) that defines the nature of the dimerization interface. Different ligands might promote slightly different domain I/III relationships, and thus impose subtly different bends on domain II.

Figure 4.

Equilibrium scheme for EGF binding to EGFR and EGFR dimerization. For ligand binding, K_1 refers to EGF association with monomeric EGFR (to form an $\mathbf{RL}$ complex, where $\mathbf{R}$ is Receptor and $\mathbf{L}$ is Ligand). K_2 refers to EGF binding to an already-dimerized EGFR ($\mathbf{R}_2$) to form a singly-occupied $\mathbf{R}_2\mathbf{L}$ dimer. K_3 refers to EGF binding to the $\mathbf{R}_2\mathbf{L}$ complex to yield a fully occupied $\mathbf{R}_2\mathbf{L}_2$ dimer. K_α denotes the association constant for free EGFR (to generate $\mathbf{R}_2$). K_β describes EGFR association with a monomeric EGF:EGFR complex to yield the $\mathbf{R}_2\mathbf{L}$ complex. K_γ reflects dimerization of the EGF:EGFR complex to form an $\mathbf{R}_2\mathbf{L}_2$ dimer. The system can be described completely with any four of the listed association constants. According to Macdonald and Pike [75] and Wofsy *et al.* [67], the concave-up Scatchard plots seen when EGF binding to cell-surface EGFR is analyzed can be explained if $K_2 > K_3$ – i.e. if EGF binds more strongly to an $\mathbf{R}_2$ dimer than to an $\mathbf{R}_2\mathbf{L}$ dimer, so that the second ligand binding event is weaker than the first. If $K_2 = K_3$, the model predicts positive cooperativity [67–69, 75]. In addition, K_2 must be greater than K_1 in order for EGF binding to drive dimerization. In models that predict negative cooperativity, the $\mathbf{R}_2\mathbf{L}$ species must accumulate, as discussed in the text. Loss of domains that stabilize the $\mathbf{R}_2\mathbf{L}$ species may explain the failure to see negative cooperativity with the isolated EGFR extracellular region. In the model of Macdonald and Pike [75], values of K_α and K_β are similar, providing a potential explanation for the significant ligand-independent EGFR dimerization described in the text. In this model, K_γ is reduced by ~10-fold, suggesting that binding of a second ligand molecule to the $\mathbf{R}_2\mathbf{L}$ species reduces the stability of the dimer. Rather than imposing negative cooperativity, Klein *et al.* [69] assumed in their analysis that the binding events represented by K_2 and K_3 were equivalent – citing the symmetric nature of the EGF:sEGFR dimeric complex. With this model, the experimentally observed concave-up Scatchard curvature can be modeled by including an ‘external site’ that stabilizes a subset of occupied EGFR dimers.