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Putting together structures of epidermal growth factor receptors

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Abstract

Numerous crystal structures have been reported for the isolated extracellular region and tyrosine kinase domain of the epidermal growth factor receptor (EGFR) and its relatives, in different states of activation and bound to a variety of inhibitors used in cancer therapy. The next challenge is to put these structures together accurately in functional models of the intact receptor in its membrane environment. The intact EGFR has been studied using electron microscopy, chemical biology methods, biochemically, and computationally. The distinct approaches yield different impressions about the structural mode of communication between extracellular and intracellular regions. They highlight possible differences between ligands, and also underline the need to understand how the receptor interacts with the membrane itself.

Introduction

Growth factor receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR) have been the subjects of intense study for many years [1,2]. There are 58 RTKs in the deduced human proteome, and all play key roles in regulating cellular processes such as proliferation, differentiation, cell survival and metabolism, cell migration, and cell cycle control [3]. Importantly, aberrant activation of RTK signaling by mutation, gene amplification, gene translocation or other mechanisms has been causally linked to cancers, diabetes, inflammation, and other diseases. These observations have prompted the development of many targeted therapies that inhibit RTKs such as EGFR [4•], Kit, VEGFR, or their ligands – typically employing therapeutic antibodies [5] or small molecule tyrosine kinase inhibitors [6].

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Conflict of interest

None declared.

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Following the initial discoveries for EGFR [7] and the platelet-derived growth factor receptor (PDGFR) [8] that ligand-stabilized dimers are essential for RTK signaling, structural studies over the past decade or so have guided development of quite sophisticated mechanistic views [1]. Each RTK has a ligand-binding extracellular region (ECR) that is linked by a single transmembrane α-helix to an intracellular tyrosine kinase domain (TKD). Structures of the isolated ECRs and TKDs from several RTKs point to surprising mechanistic diversity across the larger family [1]. Unliganded RTKs exist as an equilibrium mixture of inactive monomers, inactive dimers and active dimers (Figure 1), except for the extreme case of the insulin receptor (IR), which is covalently dimerized [9]. Extracellular ligand can bind to monomers, to inactive dimers, or to active dimers – in each case pushing the equilibria shown in Figure 1 towards the central ligand-bound active dimer. Thus, ligand binding can drive receptor dimerization (Figure 1, upper), or can promote inactive-to-active conformational transitions in dimers (Figure 1, lower). Regardless of pathway, the intracellular TKD of the ligand-stabilized dimer becomes activated either through *trans*-autophosphorylation or through induced allosteric changes [1,10]. Roles for other parts of the receptor in RTK activation, including the juxtamembrane (JM) and transmembrane (TM) segments, have also become clearer. The key current challenge for the field is to assemble data from many studies of isolated RTK parts into coherent views of how the intact receptors are regulated in their native membranes. We will focus here on recent efforts to do this for the EGFR (or ErbB receptor) family.

The missing links in intact RTKs: Flexible or rigid?

A central goal in extrapolating to the intact RTKs from studies of isolated soluble domains is to understand how the individual parts of the receptor communicate with one another. The methods that have been used to produce and study the isolated domains inevitably yield the impression that inter-domain linkers are flexible and disordered. For example, extracellular juxtamembrane regions have typically only been observed as C-terminal extensions of the soluble ECR. Similarly, intracellular juxtamembrane regions have been encountered predominantly as N-terminal extensions of TKD constructs, or as short peptides. In each of these contexts, the JM regions are incomplete, and may appear disordered and flexible simply because key structural restraints have been removed. Nonetheless, this possible artifact has strongly influenced thinking about linkages between the extra- and intra-cellular regions [11], and in turn about mechanisms of RTK signaling. Highly flexible linkages between extra- and intra-cellular regions of RTKs are fully consistent with simpler ligand-induced dimerization models for transmembrane signaling by RTKs. However, it is more difficult to understand how subtle allosteric communication across the membrane could be achieved if the linkages are truly flexible. For example, since flexible linkage implies structural independence of the extra- and intra-cellular regions, it is difficult to envision how a transition from inactive to active dimer in Figure 1 could be controlled precisely by ligand without more rigid (or restricted) connections.

Recent experimental studies with intact – or nearly intact – EGFR differ in the impressions they provide about how flexibly or rigidly the extra- and intra- cellular regions are linked. Springer's laboratory used cysteine crosslinking and mutagenesis approaches to investigate this issue for EGFR expressed in Ba/F3 cells [12]. They were unable to identify any specific

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JM or TM region interfaces that were required for EGFR signaling, leading them to argue that the linkage across the membrane is too flexible to transmit a specific orientation between the extracellular and intracellular regions. Consistent with this, negative-stain electron microscopy studies of (nearly) full-length EGFR in dodecylmaltoside micelles showed that a given extracellular dimer can be linked to several different arrangements of the intracellular kinase domain [13••,14]. Similarly, dimers driven by inhibitor binding to the intracellular TKD could couple to multiple different ECR conformations [13••]. Biochemical studies are also consistent with such structural independence of the extra- and intra-cellular regions [15,16••]. Contrasting with these observations, however, Schepartz and colleagues have reported that different precise conformations within the EGFR intracellular region can be induced by distinct activating ligands [17••]. They used a method called bipartite tetracysteine display that reports on formation of a chemically detectable tetracysteine motif when two cysteine pairs come together at the dimer interface. EGF activation of the receptor led to formation of a tetracysteine motif that requires the intracellular JM helix [18] shown in Figure 2A to form an antiparallel coiled-coil dimer (Figure 2B/C) as proposed by Kuriyan and colleagues [19,20••]. Surprisingly, transforming growth factor-α (TGFα), which also activates EGFR, did not bring these two cysteine pairs together in the same way – arguing that TGFα does not induce formation of the same intracellular antiparallel coiled coil. Instead, activation of EGFR with TGFα (but not EGF) stabilized an alternative tetracysteine motif, consistent with a different intracellular JM structure. Evidence for 'inside-out' signaling in EGFR has also been reported, where alterations in the intracellular JM region directly influence allosteric EGF binding to the ECR of the intact receptor analyzed in CHO cells [21-23]. The contradictory views of flexibility versus rigidity in linkages between the domains leave the path to understanding the intact receptor unclear, although it seems reasonable to doubt that the inactive dimers known to form in the absence of ligand [24-26] could be regulated by extracellular ligand if all linkages are always highly flexible.

Does the membrane hold the key?

All of the studies that support direct conformational communication between the extra- and intra-cellular regions of EGFR were performed in cells [17••,21,22]. By contrast, most of those that explicitly suggest otherwise were performed in detergent micelles [13••-15] – where the potentially important influences of specific membrane lipids (or membrane geometry) are absent. Studies of intact EGFR in liposomes with defined lipid compositions [27] have shown that the ganglioside GM3 inhibits ligand-independent activation (and dimerization) of the receptor, apparently through interactions with a site in its extracellular JM region. McLaughlin and colleagues [28,29] also proposed a model in which interaction of the intracellular JM region (and TKD) with anionic phospholipids in the inner leaflet of the plasma membrane (notably PtdIns(4,5)P_2) exerts an inhibitory effect that must be overcome in order for EGFR to signal. Association of the JM and TM regions with specific membrane lipids is likely to define specific structures in the linkages between extra- and intra-cellular regions of RTK that are more well-defined structurally (and potentially rigid) than is typically appreciated.

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Recent studies have begun to shed some structural light on how membrane interactions with the *intra*cellular JM region of EGFR might influence the signaling mechanism. Endres et al. [20••] found that simply tethering the complete intracellular region of EGFR to the inner leaflet of the plasma membrane maintains the TKD in a largely monomeric state and inhibits its kinase activity. Parallel computational studies [30•] suggest that this results from the previously proposed [29] inhibitory interaction of the JM and TKD regions of EGFR with the negatively-charged membrane surface. The data of Endres et al. [20••] further indicated that TM-mediated dimerization reverses this inhibitory effect. Moreover, NMR studies of a 60-residue peptide containing the TM and part of the JM region solubilized in lipid bicelles led them to conclude that specific TM dimerization through an N-terminal GxxxG motif stabilizes formation of an antiparallel coiled-coil between the two JM fragments in the dimer – the same JM coiled coil shown in Figure 2B/C that was investigated in the bipartite tetracysteine display studies of intact EGF-bound EGFR described above [17••,19]. Independent solid-state NMR studies of a similar TM-JM peptide from the EGFR relative ErbB2 in vesicles containing acidic phospholipids [31•] further suggested that an activating mutation in the TM domain leads to release of the JM region from the anionic membrane surface. Collectively, these data suggest that ligand-induced dimerization of the receptor (or reorientation of receptors within a dimer) may engage the TM domain in a specific dimer that promotes both the formation of activating interactions in the JM region and disruption of inhibitory interactions between the JM region (and possibly TKD) and the membrane surface.

Less is known about the specifics of how lipids in the extracellular leaflet of the membrane interact with and influence the ECR. Several studies have applied Förster resonance energy transfer (FRET) approaches to investigate the conformation of the ECR of intact EGFR in membranes – and how it changes upon ligand binding – as well as the relationship between the plane of the membrane surface and the long axis of the ECR [32-34]. Modeling studies have also suggested that the ECR of the receptor may associate with the membrane surface [30•,32], providing opportunities for specific lipid interactions that may influence the properties of the receptor. There has been some advance in understanding the effects of the ganglioside GM3 on EGFR activity [27,35], but structural details remain sparse and other direct effects of membrane lipids on the ECR and extracellular JM region remain to be explored.

Negative cooperativity

A key characteristic of ligand binding at the cell surface to EGFR [36], IR [37], and other receptors [38] is negative cooperativity – which is lost when soluble forms of the ECR from human EGFR [39] or IR [40] are studied in isolation. Several studies have shown that intracellular and/or transmembrane regions are required for this negative cooperativity to be manifest [21,22,40,41], implying that these parts of the receptor contribute to breaking the symmetry of the dimer – as required for the two sites to have distinct binding properties [42]. Such propagation of dimer asymmetry across the membrane would surely require defined structures in the regions that connect extra- and intracellular regions, and is difficult to reconcile with highly flexible JM linkers. It is important to note, however, that the *Drosophila* EGFR does retain negative cooperativity in binding to its ligands even when the

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ECR is studied in isolation [43] – and this has allowed the structural basis of negative cooperativity to be defined (Figure 2). In brief, binding of one ligand stabilizes a singly-liganded asymmetric dimer in which the unoccupied ligand-binding site is compromised [43]. The binding affinity of the second ligand is thus reduced, constituting a half-of-the-sites mode of negative cooperativity [44]. Leahy's group has provided important evidence consistent with a similar mechanism in the cases of human EGFR and ErbB4 [16••]. They generated receptor variants with a debilitating mutation either in the ligand-binding site or the TKD. Neither variant could signal when introduced into cells on its own, but coexpression of the two restored signaling ability, arguing that a singly-liganded dimer with only one active TKD is capable of transmembrane signaling. By comparing human ErbB receptor ECR dimer crystal structures with different bound ligands, Leahy and colleagues went on to identify two types of dimer interface [16••], a 'flush' interface that resembles the asymmetric (singly-liganded) dimer seen for the Drosophila EGFR [43] and a 'staggered' interface seen in the ECRs from EGFR (with bound EGF [12]) and ErbB4 (with bound neuregulin1β [16••]). Consistent with results from the *Drosophila* receptor [43], with models based on detailed fitting of cell-surface binding data [36], and with molecular modeling studies [30•], these observations suggest that the 'flush' interface drives the most stable dimers, which are singly liganded (Figure 2B). Binding of the second ligand is weaker, and also forces the dimer interface into the less stable 'staggered' conformation (Figure 2C). Taken together, these findings suggest both a structural basis for negative cooperativity and a possible structural distinction between singly- and doubly- liganded ErbB receptor dimers, which may signal differently to allow the nature of the signal to vary with ligand concentration [45] and possibly to provide ligand specificity in ErbB signaling [46].

It should be noted that conclusions differ as to whether the asymmetry in the intracellular regions (where the TKD is allosterically activated) must match that in the extracellular region or ErbB receptor dimers [16••,47•]. This will be an important issue to resolve – as it lies at the heart of understanding the nature of the linkage been extra- and intracellular regions. Recent computational studies have also suggested that direct association of membrane lipids with the ECR [32] and/or the receptor-bound ligands [48] may contribute to imposing the asymmetry that manifests itself as negative cooperativity. These calculations underline further the need to understand interactions between the receptor and the membrane.

A model for EGFR activation

The model shown in Figure 2 summarizes key proposed steps in activation of human EGFR. In the absence of ligand, the ECR exists in a tethered conformation with the domain II 'dimerization arm' engaged in an intramolecular interaction with domain IV that occludes the dimer interface [49]. The TKDs and the N-terminal portions of each intracellular JM region are thought to be engaged in autoinhibitory interactions with the membrane surface [20••,28-30•]. Following ligand binding, it appears likely that a singly-liganded dimer of the ECR can form [16••,36], using the 'flush' dimer interface observed in a crystal structure of the asymmetric singly-liganded ECR from *Drosophila* EGFR [43]. As a result of ligand-induced ECR dimerization, the TKDs also dimerize, forming the activating asymmetric dimer identified by Kuriyan and colleagues [50]. The intracellular JM region dissociates

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from the membrane surface [20••,31•], and contributes directly to receptor dimerization both by 'cradling' the C-lobe of its neighbor [18,19] and through formation of the antiparallel coiled coil seen in NMR studies [20••] and inferred using bipartite tetracysteine display [17••]. As discussed in this article, it remains unclear whether the asymmetry in the extracellular region of this singly-liganded dimer has a fixed relationship to that in the intracellular region [16••,47•]. This is a central issue for understanding how EGFR functions. Most studies suggest a flexible linkage [12-15], but key questions still remain. The second ligand binding event, leading to the doubly-liganded dimer, is thought to induce a transition from a 'flush' to a 'staggered' dimerization interface that is more symmetric [16••,30•], and may actually be weaker – possibly allowing subunit exchange. We do not include the inactive dimer species from Figure 1 in this more detailed model, since its nature is unclear. Modeling studies have argued that a domain II-mediated dimer can exist in the absence of ligand [30•,32], but there is no experimental evidence that this type of unliganded ECR dimer is independently stable for the human receptor. It has only been observed for the *Drosophila* receptor [43]. Models of specific unliganded dimeric structures have led to the suggestion that the ECR serves to inhibit dimerization driven by the JM and TKD regions in the absence of ligand [20••,30•] – and that ligand binding serves to relieve this inhibition. This hypothesis seems unlikely to be correct, since the ECR appears to form no specific dimer in the absence of ligand [12,13••], yet dimerizes quite strongly once ligand is bound [39]. It therefore seems more likely that the ECR provides a substantial proportion of the driving force for ligand-induced receptor dimerization (rather than playing a passive role). Indeed, if ligand binding activates EGFR simply by removing an ECR-mediated impedance to TKD dimerization, it would be difficult to understand why single amino acid substitutions in the dimerization interface block EGF-induced signaling [51,52], and conversely why mutations that destabilize the tethered configuration are not activating [53,54].

Conclusions

Our mechanistic understanding of EGFR and its relatives has advanced dramatically in recent years, and the past year or two has seen substantial progress in putting the results of studies with isolated domains together into initial views of how the intact receptor works. New insights into the origin of allosteric regulation of EGFR have been gained through a combination of innovative structural, biochemical, cellular, and computational studies. A self-consistent picture is beginning to emerge. Two key issues remain unclear, however, and represent the current frontiers in studies of EGFR. The first – for which we describe progress in this review – centers on the influence of specific interactions of the receptor with membrane lipids, which seem likely to define the structural 'connections' between extra- and intra-cellular regions of the receptor. The second centers on the role of the carboxy-terminal ~230 amino acids, which is believed to play a regulatory role for which little detail has so far been defined [55•].

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• of special interest

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structure of the neuregulin-bound ErbB4 extracellular region (presented here) suggest two types of dimer interface - 'flush' and 'staggered', consistent with the model for negative cooperativity proposed in reference 43 for the Drosophila receptor. These observations led the authors to investigate (using mutated receptors) whether singly-liganded EGFR (or ErbB4) dimers could signal. The results suggest that the singly liganded receptors can signal, suggesting that the molecular basis for negative cooperativity is similar in human and invertebrate EGFRs. [PubMed: 22699492]

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Highlights

- Several studies suggest flexible linkage between extra- and intracellular regions
- Others imply more rigid connections, required for allosteric regulation of dimers
- Interactions with membrane lipids play important roles in EGFR regulation
- Cellular studies suggest half-of-the-sites negative cooperativity for human EGFR

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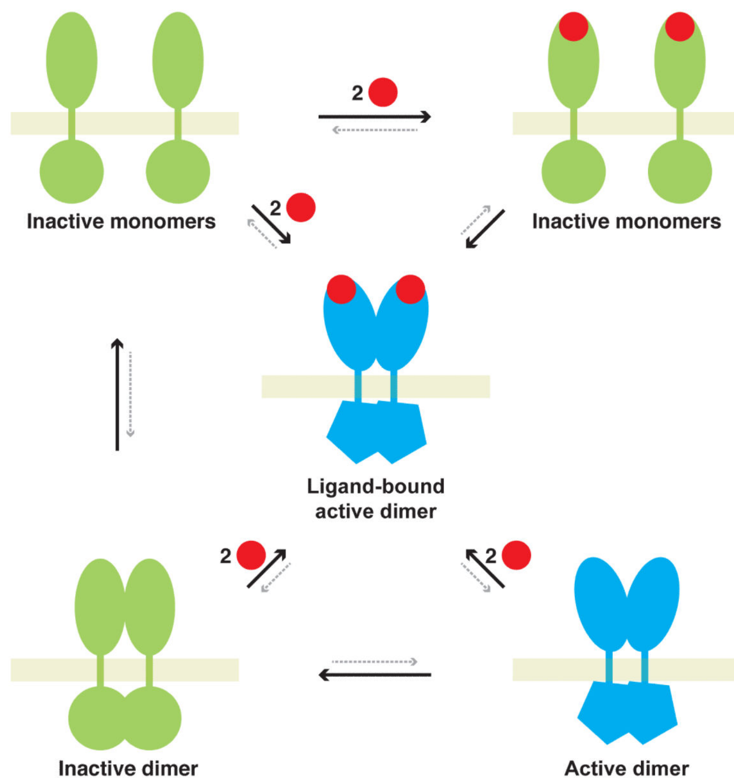

Figure 1.
Equilibria involved in activation of RTKs with the EGF receptor as an example. Inactive species are green, active species blue. Inactive monomers are in equilibrium with inactive dimers, the structural nature of which remains unclear. These in turn are in equilibrium with active dimers. Ligand binding activates the receptor by driving dimerization of inactive monomers, by inducing conformational changes in inactive dimers [24], or simply by stabilizing active dimers.

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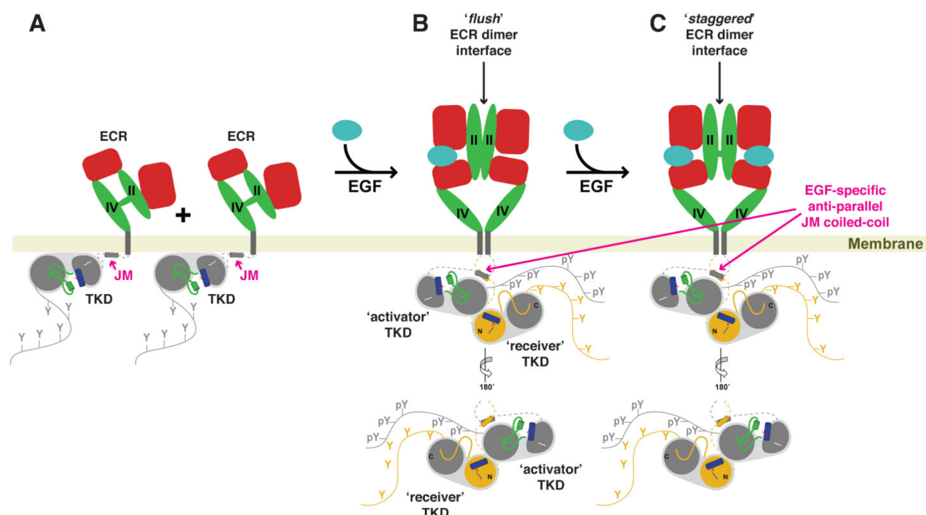

Figure 2.

More detailed view of EGF-induced activation of EGFR, as described in text. In the absence of ligand (**A**), the ECR adopts a tethered conformation, with an autoinhibitory tether interaction between domains II and IV. The TKD and JM regions lie against the membrane, making what are believed to be additional autoinhibitory interactions. Domains I and III of the ECR are colored red, and domains II and IV are green. The JM helix is shown as a short cylinder and labeled in magenta. The N-and C-lobes of the kinase are also labeled, and both helix αC (blue) and the short helix in the activation loop (green) that interacts with αC to inhibit the TKD [50] are shown. The C-tail is also depicted as a curve bearing 5 tyrosines. As described in the text, binding of a single ligand (**B**) induces formation of a singly-liganded dimer with a 'flush' (presumed asymmetric) ECR dimer interface. The JM region forms an anti-parallel helix, as labeled in magenta, and the TKDs form an asymmetric dimer in which the activator (grey) allosterically activates the receiver (shown with an amber N-lobe). It is not clear how the extra- and intracellular asymmetry is structurally related, if at all. Finally, a second ligand binds to yield a more symmetric dimer with the 'staggered' ECR interface (**C**) described in the text.