The EGFR Family: Not So Prototypical Receptor Tyrosine Kinases

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The epidermal growth factor receptor (EGFR) was among the first receptor tyrosine kinases (RTKs) for which ligand binding was studied and for which the importance of ligand-induced dimerization was established. As a result, EGFR and its relatives have frequently been termed “prototypical” RTKs. Many years of mechanistic studies, however, have revealed that—far from being prototypical—the EGFR family is quite unique. As we discuss in this review, the EGFR family uses a distinctive “receptor-mediated” dimerization mechanism, with ligand binding inducing a dramatic conformational change that exposes a dimerization arm. Intracellular kinase domain regulation in this family is also unique, being driven by allosteric changes induced by asymmetric dimer formation rather than the more typical activation-loop phosphorylation. EGFR family members also distinguish themselves from other RTKs in having an intracellular juxtamembrane (JM) domain that activates (rather than autoinhibits) the receptor and a very large carboxy-terminal tail that contains autophosphorylation sites and serves an autoregulatory function. We discuss recent advances in mechanistic aspects of all of these components of EGFR family members, attempting to integrate them into a view of how RTKs in this important class are regulated at the cell surface.

The epidermal growth factor receptor (EGFR) is often considered the “prototypical” receptor tyrosine kinase (RTK) and has been intensively studied. It is one of a family of four RTKs in humans, the others being ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4 (Fig. 1). EGFR and its relatives are known oncogenic drivers in cancers such as lung cancer (Mok 2011), breast cancer (Arteaga et al. 2011), and glioblastoma (Libermann et al. 1985; Lee et al.

2006a; Vivanco et al. 2012), and inhibitors of these receptors have been among the most successful examples of targeted cancer therapies to date (Arteaga 2003; Moasser 2007; Zhang et al. 2007), including antibody therapeutics (e.g., trastuzumab and cetuximab) and small-molecule tyrosine kinase inhibitors (e.g., erlotinib, gefitinib, lapatinib).

Far from being prototypical, however, it is now clear that regulation of EGFR family mem-

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Figure 1. Schematic representation of EGFR/ErbB family receptors and their ligands. (A) The domain composition of human EGFR is shown. The extracellular region contains four domains: Domain I (amino acids 1–165), domain II (amino acids 165–310), domain III (amino acids 310–480), and domain IV (amino acids 480–620). Domains I and III are closely related in sequence, as are domains II and IV. Shown are representations of the structures of domains I and IV. Domain IV contains two types of disulfide-bonded module (C1 and C2). In C1 domains, a single disulfide constrains an intervening bow-like loop. In C2 modules, two disulfides link four successive cysteines in the patterns C1–C3 and C2–C4 to give a knot-like structure. A short extracellular juxtamembrane (eJM) region separates the extracellular region from the ~23-amino-acid transmembrane (TM) domain. Within the cell, a short intracellular juxtamembrane (iJM) region separates the tyrosine kinase domain (TKD) from the membrane. A representative EGFR TKD structure is shown. The TKD is followed by a carboxy-terminal largely unstructured tail (amino acids 953–1186) that contains at least five tyrosine autophosphorylation sites. (B) EGFR is one of four members of the EGFR/ErbB family in humans. The other members are ErbB2/HER2, for which no soluble activating ligand is shown; ErbB3/HER3, which has a significantly impaired kinase domain (Jura et al. 2009b; Shi et al. 2010); and ErbB4/HER4. The primary active moiety of the ligands for these receptors is the EGF-like domain, shown as a cartoon structure (*top right*). EGFR is activated by the EGFR agonists: EGF itself, TGF- α (transforming growth factor α), ARG (amphiregulin), and EGN (epigen). The bispecific ligands regulate both EGFR and ErbB4: HB-EGF (heparin-binding EGF-like growth factor), EPR (epiregulin), and BTC (betacellulin). Neuregulins (NRGs) 1 and 2 regulate ErbB3 and ErbB4, whereas NRG3 and NRG4 appear to be specific for ErbB4 (Wilson et al. 2009).

bers is unique among RTKs (Ferguson 2008; Lemmon 2009; Lemmon and Schlessinger 2010). Structural studies have revealed how the ~620-amino-acid isolated extracellular region is induced to dimerize after growth factor binding (Burgess et al. 2003) and how the isolated intracellular tyrosine kinase domain (TKD) becomes allosterically activated after forming an

asymmetric dimer (Zhang et al. 2006; Jura et al. 2009a; Red Brewer et al. 2009). These findings have typically been interpreted in the context of a model in which EGF family receptors are regulated through ligand-induced receptor homodimerization or heterodimerization, with growth factor binding converting the receptor from an inactive monomeric configuration to

an active dimeric conformation (Yarden and Schlessinger 1987; Schlessinger 1988, 2014; Ullrich and Schlessinger 1990). Although this original model has stood the test of time and initiated a whole field of studies of ligand-induced RTK dimerization, recent work has provided highly sophisticated views of a unique mode of allosteric regulation used by EGFR.

ErbB RECEPTORS AND THEIR LIGANDS

Although EGFR/ErbB family members are most frequently considered in the context of human cancer—where EGFR, ErbB2, and ErbB3 are validated therapeutic targets—the biology of these broadly expressed receptors is very complicated (Yarden and Sliwkowski 2001; Burgess 2008). The effect of knocking out the *EGFR* gene in mice ranges from embryonic lethality in one genetic background to death at birth in another, to postnatal death in yet another (Sibilia et al. 2007). Defects are seen in bone, brain, heart, and various epithelia—notably skin, hair, eyes, and lungs. Mouse knockouts of ErbB2, ErbB3, or ErbB4 are all embryonic lethal, also with neurodevelopmental and cardiac defects in each case (Sibilia et al. 2007; Burgess 2008). It is now clear that ErbB 2/3/4 signaling has a key role in both cardiac development and maintenance of cardiac function in the adult (Pentassuglia and Sawyer 2009).

EGFR is regulated by at least seven different activating ligands in humans (Harris et al. 2003; Schneider and Wolf 2009) listed in Figure 1: EGF itself, transforming growth factor α (TGF- α), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (ARG), epiregulin (EPR), and epigen (EGN). Each contains an EGF-like domain that is responsible for receptor binding and activation, with a characteristic pattern of six spatially conserved cysteines (that form three intramolecular disulfides). The EGFR ligands are all produced as membrane-bound precursor proteins (Harris et al. 2003) and are cleaved by cell-surface proteases to yield the active growth factor species as described by Adrain and Freeman (2014). Although defects in EGFR affect a wide range of processes, it remains unclear which ligands are

responsible in which context—with a few exceptions (Fiske et al. 2009). ErbB3 and ErbB4 are regulated by neuregulins (NRGs) (Falls 2003)—also called heregulins (HRGs), a family of ligands produced from four genes (*NRG1*—*NRG4*) in a wide variety of different isoforms that all contain an EGF-like domain. NRG1 and NRG2 bind both ErbB3 and ErbB4, whereas NRG3 and NRG4 appear to be ErbB4 specific (Wilson et al. 2009). The NRGs and their receptors have an important role in nervous system development (Birchmeier 2009). NRG1 and ErbB4 have been linked to schizophrenia (Rico and Marín 2011). Three of the EGFR ligands mentioned above (BTC, EPR, and HB-EGF) also bind and activate ErbB4 and are termed “bispecific” ligands (Riese and Stern 1998; Wilson et al. 2009). For ErbB2, no soluble ligand has been identified despite a great deal of investigation in the 1990s. This orphan receptor is generally assumed only to be regulated by heterodimerization with other ErbB family receptors, although its close structural resemblance to the *Drosophila melanogaster* EGFR suggests that membrane-associated ErbB2 ligands may remain to be discovered (Alvarado et al. 2009).

Each ErbB ligand is produced as a membrane-bound precursor that is processed in a ligand-specific manner (Buonanno and Fischbach 2001). Although the EGF-like domains of the NRGs and the EGFR ligands appear to be sufficient for much of their biological effect, it is clear that other parts of the full-length ligands *do* influence signaling—although in ways that are not yet fully understood. The discussion in this review is limited to receptor activation by the EGF-like domains. The receptors themselves (Fig. 1) all have a large extracellular region of ~ 620 amino acids that can be subdivided into four domains. Domains I and III are related to one another (and to similar domains in the insulin receptor) and serve as the primary ligand-binding regions. Domains II and IV are cysteine-rich domains that share similarities with laminin repeats (Ward et al. 1995) and contain a string of disulfide-bonded modules. A single transmembrane domain links the extracellular region to the ~ 540 -amino-acid intracellular region of the receptor that contains a tyrosine

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kinase domain as well as a carboxy-terminal “tail” of ~230 amino acids. Binding of activating ligands to EGFR or ErbB4 promotes strong homodimerization of these receptors (Ferguson et al. 2000). In addition, it is thought that the four members of the family form various heterodimers (Yarden and Sliwkowski 2001). In particular, ErbB2 and ErbB3—which do not form homodimers (Ferguson et al. 2000; Cho et al. 2003; Berger et al. 2004)—signal only through heterodimerization (with one another and with other ErbB receptors). Following activation, a series of tyrosines in the carboxy-terminal tail become autophosphorylated *trans* (Honegger et al. 1989) and serve as “docking” sites for phosphotyrosine-binding SH2 and PTB domains as discussed in Wagner et al. (2013). Different ErbB family members harbor different complements of tyrosine phosphorylation sites in their carboxy-terminal tails (Lemmon and Schlessinger 1994; Yarden and Sliwkowski 2001; Jones et al. 2006), thus eliciting distinct but overlapping sets of responses.

STRUCTURAL BASIS FOR LIGAND-INDUCED DIMERIZATION OF ErbB RECEPTORS

A series of crystal structures published in 2002 and 2003 provided a dramatic leap forward in our understanding of transmembrane signaling by ErbB receptors (Burgess et al. 2003) and laid the foundation for much of our current understanding of how these receptors function. Structures of the EGFR extracellular region with and without bound growth factor provided a satisfying, and unexpected, model for ligand-induced receptor dimerization. They showed that—in contrast with other RTKs—dimerization of the EGFR extracellular region is mediated entirely by receptor–receptor contacts. With the exception of the insulin receptor, this makes EGFR and its relatives unique among the RTKs discussed in this collection. In all other cases that are understood, the activating growth factor ligand binds at the dimer interface, effectively serving to cross-link the two receptors into a dimer (Lemmon and Schlessinger 2010)—typ-

ically with the aid of additional receptor–receptor contacts. As shown on the right side of Figure 2, the bound ligands in an EGFR dimer are as far as they possibly could be from the dimer interface (Garrett et al. 2002; Ogiso et al. 2002)—and, indeed, from one another. Each bound ligand is bivalent but simultaneously contacts two points (domains I and III) in the extracellular region of a single receptor molecule, rather than spanning two receptors. Domains I and III are rigid β -helix/solenoid structures that do not change conformation after ligand binding (Ferguson 2004). Dimerization is driven almost exclusively by a “dimerization arm” that projects from domain II, although inter-receptor domain IV contacts (Fig. 2A) may also contribute weakly (Dawson et al. 2005; Lu et al. 2010). Activation (by way of inducing dimerization) involves an unexpectedly large ligand-induced conformational change that exposes this domain II dimerization arm. In the absence of bound ligand, the EGFR, ErbB3, and ErbB4 extracellular regions form the “tethered” structure shown on the left side of Figure 2A, in which the domain II dimerization arm is buried by intramolecular interactions with domain IV (Cho and Leahy 2002; Ferguson et al. 2003; Bouyain et al. 2005). Ligand binding to domains I and III effectively pulls these domains together and “extends” the receptor’s extracellular region to break the domain II/IV tether and expose the dimerization site—yielding a dimerization-competent ligand-bound EGFR extracellular region that self-associates strongly.

ACTIVATION OF THE KINASE DOMAIN BY FORMATION OF AN ASYMMETRIC DIMER

The first crystallographic view of the EGFR TKD (Stamos et al. 2002) confirmed previous reports that activation-loop phosphorylation is not required for it to adopt an active-like structure (Gotoh et al. 1992; Burgess et al. 2003) but provided little insight into how it is activated by receptor dimerization. The likely activation mechanism was revealed in 2006 through insightful analysis of additional crystal structures

Figure 2. Basic model for EGF-induced dimerization and activation of EGFR. (A) In the absence of bound EGF, the human receptor is largely monomeric. The intracellular TKD is inactive, and the extracellular region adopts a “tethered” configuration in which a β -hairpin from domain II (the dimerization arm) forms intramolecular autoinhibitory interactions with domain IV. EGF binds to both domains I and III and induces a dramatic conformational change that “extends” the extracellular region and exposes the dimerization arm. With the domain II dimerization arm exposed, the EGFR extracellular region dimerizes (Burgess et al. 2003), bringing the intracellular TKDs into close proximity so that they can form the asymmetric dimer that leads to kinase activation (Zhang et al. 2006). In the asymmetric dimer, one TKD (gray) serves as the “activator,” and the other (cyan) is the “receiver” that becomes allosterically activated and *trans*-phosphorylates tyrosines in the tail of the activator. (B) A cartoon representation of the structural changes shown in (A). (From Ferguson 2008; adapted, with permission, from the author.)

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of the active EGFR TKD by the Kuriyan laboratory (Zhang et al. 2006). A characteristic asymmetric dimer was seen in the crystals used to solve each of these structures, in which the carboxy lobe of one TKD abuts the amino lobe of the other TKD, as depicted in the right-hand sides of Figure 2A and B. This relationship resembles that seen between cyclins and the amino lobe of the cyclin-dependent kinases (CDKs) that they activate, and the carboxy lobe of the “activator” kinase in an EGFR TKD dimer is thought to induce allosteric changes in the amino lobe of the “receiver” kinase and thus activate it. Analysis of a series of mutations in the asymmetric dimer interface confirmed its importance for activation of intact EGFR (Zhang et al. 2006), and subsequent studies showed that the NRG receptor ErbB4 is regulated in much the same way (Qiu et al. 2008).

These studies revealed that just as ErbB receptors are anything but prototypical RTKs in terms of their extracellular regulation, so are they unique in their intracellular regulation. Whereas activation-loop phosphorylation is a key step in regulating the TKDs of most RTKs (see Belov and Mohammadi 2013; Heldin and Lennartsson 2013; Hubbard 2013), it plays no part in the ErbB family (Gotoh et al. 1992; Burgess et al. 2003; Jura et al. 2011). Consistent with this distinction, the “inactive” conformation of ErbB family TKDs differs substantially from that seen in other RTKs (Wood et al. 2004; Zhang et al. 2006) and instead resembles inactive Src family kinases and CDKs. The model for allosteric activation of the EGFR TKD by its asymmetric dimerization following extracellular ligand binding provides a satisfying explanation for how this receptor can be regulated without activation-loop phosphorylation. In brief, the carboxy lobe of the activator kinase interacts with the amino lobe of the receiver and induces several conformational changes in the receiver amino lobe (Fig. 3A,B). In particular, helix α H of the activator interacts directly with helix α C of the receiver, causing the α C helix to rotate from its “out” position in the inactive EGFR TKD to the “in” position seen in active kinases. This movement disrupts interactions between the α C helix and the short helix in the activation

loop—freeing the activation loop to adopt the conformation typically seen in active kinases. In addition, rotation of helix α C brings a key glutamate in this helix (E738 in mature receptor numbering) close to the side chain of lysine 721, with which it can form a salt bridge to promote ATP binding. Thus, interaction with the carboxy lobe of the activator promotes an inactive-to-active conformational transition in the receiver TKD (Zhang et al. 2006), and the EGFR kinase is activated allosterically rather than through activation-loop phosphorylation. The models in Figures 2 and 3B raise the question as to whether this is “vectorial,” with only one EGFR becoming autophosphorylated in its carboxy-terminal tail. Presumably, however, the two molecules in the dimer dissociate and reassociate sufficiently rapidly that both can occupy the activator position (and thus be *trans*-autophosphorylated) for at least part of the time—although it should be noted that *cis*-autophosphorylation has not been formally excluded as a possibility.

EGFR TKD MUTATIONS IN LUNG CANCER

Importantly, the allosteric activation mechanism described above provides a satisfying explanation for the effects of oncogenic driver mutations in the EGFR TKD found in ~10% of non-small-cell lung cancer patients (Sharma et al. 2007). These mutations were identified in 2004 in lung cancer patients who responded dramatically to the EGFR tyrosine kinase inhibitors (TKIs) erlotinib and gefitinib (Lynch et al. 2004; Paez et al. 2004; Pao et al. 2004). Many of these mutations—notably those at L834 and L837 (Fig. 3A)—occur at residues that stabilize “autoinhibitory” interactions in the inactive EGFR TKD. In particular, mutations such as L834R and L837Q disrupt autoinhibitory interactions between an α -helix in the activation loop (green in Fig. 3A; seen only in the inactive TKD) and the α C helix (blue in Fig. 3A) that hold the α C helix away from the position that it adopts in the active kinase. When this autoinhibitory interaction is disrupted, the inactive TKD conformation is destabilized, leading to its constitutive activation and the acquisition of ligand-independent (oncogenic) signaling properties

Figure 3. Intracellular EGFR activation. (A) EGFR TKD is shown in its active (left: PDB entry 2GS6) and inactive (right: PDB entry 2GS7) configurations (Zhang et al. 2006). The amino and carboxy lobes are marked, and the nucleotide moiety is shown in stick representation. The α C helix (dark blue) occupies the "in" position in the active TKD and the "out" position in the inactive TKD. As a result, the E738 side chain is brought sufficiently close to the K721 side chain to form a salt bridge only in the active TKD. An additional short α -helix forms in the activation loop only in the inactive TKD (green) and interacts with the α C helix to promote its displacement. Mutations at two residues in this short A-loop helix (L834 and L837 in mature EGFR, L858 and L861 in pro-EGFR) are among the most common seen in EGFR-driven non-small-cell lung cancer (Sharma et al. 2007). Y845 (labeled) in the A-loop is equivalent to the key site of activating autophosphorylation in other RTKs, but its phosphorylation is not required for EGFR activation. (B) A model of an EGFR TKD asymmetric dimer, based on crystal packing in the 2GS6 structure (Zhang et al. 2006) in which an inactive TKD (from 2GS7) has been modeled in the activator position. The activator (gray) remains inactive in this discrete asymmetric dimer, and the receiver (cyan) becomes activated. (C) The same asymmetric dimer shown in (B) is shown linked to the TM domain based on a composite view of the TM and iJM structure derived from crystallographic, NMR, and computational studies (Jura et al. 2009a; Red Brewer et al. 2009; Endres et al. 2013). The TM domain forms a symmetric dimer mediated by its more amino-terminal GxxxG motif, and this, in turn, is thought to drive formation of an antiparallel dimer between helices in the amino-terminal part of the iJM region (magenta). The remainder of the iJM region in the receiver "cradles" the carboxy lobe of the activator to form the iJM "latch" that stabilizes the activating asymmetric dimer. The structure of the iJM latch region in the activator is not defined. (D) Close-up view of the iJM latch cradling the carboxy lobe of the activator TKD. Mutations at L664, V665, P675, L680, I682, and other residues in the latch impair EGFR activation, and a V665M mutation is activating (Jura et al. 2009a; Red Brewer et al. 2009).

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by the EGF receptor. Numerous structures of mutated EGFR TKD variants found in cancer patients have provided a rich understanding of how somatic mutations activate the receptor to become oncogenic drivers, as reviewed elsewhere (Eck and Yun 2010). Combined with biochemical analysis, these studies have also shown how secondary mutations can promote drug resistance and have provided important insight into the requirements for “next-generation” EGFR-targeted TKIs (Ohashi et al. 2013).

PIECING TOGETHER INTACT ErbB RECEPTORS THROUGH EXPERIMENT

The Transmembrane (TM) Domain

Although the models presented in Figures 2 and 3B likely capture many of the key interactions involved in EGFR regulation, they are clearly missing several important elements. For example, it has been known for some time that the single transmembrane (TM) domain of each ErbB family member self-associates in lipid membranes (Mendrola et al. 2002) through one or more GxxxG dimerization motifs (Lemmon et al. 1994). Cysteine cross-linking studies in cell membranes suggest that the two TM domains in an EGF-stimulated EGFR dimer contact one another in the more amino terminal of two GxxxG-motif regions (Lu et al. 2010). However, EGFR signaling appears to be highly resilient to mutations in the TM domain of the receptor when ligand-induced autophosphorylation is assessed in qualitative and semiquantitative studies (Kashles et al. 1988; Carpenter et al. 1991; Lu et al. 2010). Moreover, an EGFR variant with its TM domain replaced with poly-leucine retains signaling activity in typical cell-based assays of this sort (JM Mendrola and MA Lemmon, unpubl.). Such observations led Springer and colleagues to argue that the intracellular and extracellular regions of EGFR are “loosely” linked (Lu et al. 2010)—and that no specific transmembrane (or extracellular juxta-membrane) interfaces are required for EGF-induced autophosphorylation of EGFR. However, more quantitative immunofluorescence-based

studies of EGFR autophosphorylation have recently indicated that the TM domain contributes to EGFR dimerization (and activation). An NMR-guided structural model suggested that the EGFR TM region dimerizes through the more amino terminal of its two GxxxG motif regions (Endres et al. 2013), as shown in Figure 3C. Interestingly, this region has the sequence TGMVGA, which contains two overlapping G/smallxxxG/small motifs as defined (Russ and Engelman 2000): TxxxG and GxxxA. Consistent with structural “plasticity” in this region, no effect on EGFR signaling was seen if either GxxxG motif was mutated individually. Replacing all four “small” residues with isoleucines, however, thus disrupting both motifs, resulted in some (~50%) reduction in EGF-induced EGFR autophosphorylation (Endres et al. 2013).

The Extracellular Juxtamembrane (eJM) Region

There is relatively little information on the properties of, and interactions mediated by, the extracellular juxtamembrane (eJM) region of EGFR family members. This is a short stretch of polypeptide, with just seven amino acids separating the most carboxy-terminal residue of domain IV seen in crystal structures of the EGFR extracellular region (Ferguson et al. 2003; Lu et al. 2010) and the hydrophobic region of the TM domain. Cysteine cross-linking studies suggest that the eJM regions of the two receptors in an EGF-induced dimer come into close proximity, but do not indicate a specific or well-defined mode of association (Lu et al. 2010). Intriguingly, however, inserting an additional flexible linker of 20–40 residues into this region appears to promote ligand-independent activation of EGFR (Sorokin 1995; Endres et al. 2013). This result might be taken to suggest that introducing flexibility between the extracellular and TM regions of EGFR leads to constitutive activation and/or that the spatial relationship between the extracellular region and the membrane is important. The eJM region of EGFR has also been suggested as the site of interaction with the ganglioside GM3, which inhibits

allosteric activation of the receptor (Coskun et al. 2011). Mutation of a lysine in the middle of the seven-residue JM linker abolishes GM3's inhibitory effect.

Differences in the eJM region can profoundly affect ErbB receptor function. ErbB4 exists as two isoforms (JM-a and JM-b) that differ in their eJM region (Elenius et al. 1997). The JM-a isoform is cleaved extracellularly following NRG binding, and is then cleaved again to generate an intracellular soluble species that is translocated to the nucleus (see Carpenter and Liao 2013; Song et al. 2013). In contrast, the JM-b isoform functions only as a membrane-bound receptor. Similar variation is not seen for the other ErbB family members.

The Intracellular Juxtamembrane (iJM) Region

Recent work has shed important light on the intracellular JM (iJM) region of EGFR and its relatives. As with other aspects, increased understanding of the iJM region has shown EGFR to be anything but a prototype for other RTKs. Whereas most studies of RTK iJM regions have shown them to have an autoinhibitory role (Hubbard 2004), it is now clear that the iJM of EGFR has a key positive role in receptor activation. This observation was first reported by Graham Carpenter's laboratory (Thiel and Carpenter 2007). They found that an intact iJM region is required for EGF-induced autophosphorylation of EGFR and is primarily required in the "receiver" kinase shown in Figures 2 and 3B. Subsequent alanine-scanning mutagenesis studies defined an activation domain in the iJM region (Red Brewer et al. 2009), extending from L664 to I682. Residues G672–I682 were already known to lie in the "amino-terminal extension" of the EGFR TKD (Zhang et al. 2006) and to play a key part in the asymmetric dimer interface that is responsible for EGFR activation. Mutating residues 675, 680, or 682 in this region disrupts the asymmetric dimer (Zhang et al. 2006). Crystallographic studies of an EGFR TKD variant containing the complete iJM showed that, in the asymmetric dimer, a region beyond the amino-terminal extension of

the receiver TKD "cradles" the carboxy lobe of the activator (Fig. 3C,D), apparently stabilizing asymmetric interactions between the two TKDs (Red Brewer et al. 2009). A similar arrangement was also reported in asymmetric ErbB4 TKD dimers (Wood et al. 2008). Based on these structures, residues 664–672 are considered to constitute a "juxtamembrane latch" (Jura et al. 2009a), enhancing EGFR activation by promoting formation of the asymmetric dimer. Interestingly, phosphorylation of a threonine (T669) in the juxtamembrane latch (by MAP kinase) modulates EGFR activation and down-regulation (Morrison et al. 1993; Li et al. 2008), and mutation of V665 to methionine—which would be predicted to strengthen the latch (Fig. 3D)—appears to be an oncogenic driver mutation in non-small-cell lung cancer (Red Brewer et al. 2009).

The juxtamembrane latch constitutes about half of the iJM region of EGFR and is preceded by an α -helix that extends from T654, an inhibitory PKC site (Welsh et al. 1991), to L664 (Red Brewer et al. 2009). Deletion of this region—even when the juxtamembrane latch is intact—impairs EGFR activation (Thiel and Carpenter 2007; Jura et al. 2009a). Intriguingly, this region appears to form an antiparallel helical dimer in the NMR-guided structural model of the TM domain plus iJM helix in Figure 3C (Endres et al. 2013). A chemical biology approach using bipartite tetracysteine display has also provided evidence for formation of this antiparallel helical dimer in the intact receptor in cells (Scheck et al. 2012). Formation of such a dimer offers a way to break the artifactual "daisy chain" seen in crystals of the isolated EGFR TKD, in which the iJM region of each molecule cradles the carboxy lobe of its neighbor, and each TKD simultaneously acts as activator and receiver. As shown in the hypothetical model presented in Figure 3C, the TM-domain dimer appears to promote antiparallel association of the iJM helices of two TKDs. Formation of this dimer displaces the remainder of the iJM in the activator TKD to break the daisy chain seen in crystals while allowing the iJM of the receiver to function as the juxtamembrane latch shown in Figure 3C,D.

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The models described above, and depicted in Figure 3, consider the TM and JM regions in only the activated EGFR. By analogy with the extracellular region and TKD, however, it is likely that these are subject to autoinhibitory interactions in the absence of ligand. Indeed, the second GxxxG motif in the TM domain may offer an alternative mode of TM packing in much-discussed preformed dimers of EGFR, which has been proposed to be autoinhibitory (Endres et al. 2013). Reorientation of the extracellular regions after ligand binding is presumed to disrupt these putative autoinhibitory TM-domain interactions. Turning attention to the iJM region, McLaughlin and colleagues (2005) made an intriguing proposal that EGFR autoinhibition involves binding of both a basic region within the iJM (including the iJM α -helix) and a basic patch on the TKD surface to anionic phospholipids in the plasma membrane inner leaflet. They showed that a peptide corresponding to the amino-terminal iJM region (residues 645–660) binds strongly to negatively charged membrane surfaces. Although they initially suggested that calmodulin binding to the iJM region might dissociate it from the membrane (and thus activate EGFR), more recent NMR studies have suggested that TM-domain dimerization or reorientation may be sufficient to dissociate the iJM region from the membrane, presumably by stabilizing the antiparallel α -helix dimer shown in Figure 3C (Matsushita et al. 2013). These possibilities clearly need further investigation in the context of the intact receptor.

The Carboxy-Terminal Tail

The $\sim$ 230-amino-acid carboxy-terminal tail of EGFR (residues 956–1186) and its relatives accounts for $\sim$ 20% of the receptor molecule. This region contains all of the known autophosphorylation sites in EGFR (except Y845 in the TKD) but is poorly characterized and is generally viewed as an unstructured region to which multiple SH2 domains bind (Gajiwala 2013). Fluorescence anisotropy and fluorescence resonance energy transfer (FRET) studies have clearly shown changes in the conformation and dynamics of the EGFR carboxy-terminal tail after

autophosphorylation (Lee and Koland 2005; Lee et al. 2006b). Moreover, truncation mutants in this region have shown altered kinase activity toward cellular substrates, leading to the suggestion that some regions within the carboxy-terminal tail function as autoinhibitory domains (requiring tyrosine phosphorylation to release the inhibition) and others as positive modulators of activity (Walton et al. 1990; Alvarez et al. 1995). A regulatory function for the region of the carboxy-terminal tail closest to the kinase domain is also supported by crystallographic studies. A structure of EGFR TKD in its inactive conformation (Wood et al. 2004) showed two TKD-proximal regions of the carboxy-terminal tail packed against the amino lobe of the kinase in the manner expected if they were to play an autoinhibitory part (Fig. 4A). Residues 971–980 form a short α -helix that lies adjacent to the hinge region between the TKD amino and carboxy lobes. Residues 986–994 form another partly helical region that contacts elements of strands β 3– β 5 in the TKD amino lobe. Interestingly, Y992 (a known major autophosphorylation site in EGFR) and Y974 (at which EGF-induced phosphorylation promotes endocytosis) (Tong et al. 2009) are positioned to stabilize these potentially autoinhibitory intramolecular interactions. The short helix that contains Y992 was also seen in a very similar location and orientation in another crystal structure of inactive EGFR TKD (Zhang et al. 2006) but is also seen (only slightly shifted) in many active EGFR TKD structures (Fig. 4B). In a symmetric dimer of inactive EGFR TKD (Jura et al. 2009a), the intramolecularly associated 986–994 helix is replaced by a longer helix (with the opposite orientation) involving residues 967–978 of a neighboring molecule. Several other locations and orientations of these regions of the carboxy-terminal EGFR TKD tail have now been seen in other crystal structures (Gajiwala 2013). Although a self-consistent picture of the mechanism of autoinhibition by the carboxy-terminal tail (and how it might be reversed by autophosphorylation) has yet to emerge, it seems highly likely that these interactions involving the carboxy-terminal tail represent an additional important regulatory element.

Figure 4. Location of the small section of the EGFR carboxy-terminal tail with known structure. (A) A structure of the inactive EGFR TKD (PDB entry 1XKK) shows that two TKD-proximal regions of the carboxy-terminal tail form short helices (colored orange) that pack against the amino lobe as described in the text. Tyrosines 974 and 992 are marked, phosphorylation of which could be involved in EGFR regulation. (B) Structures of the active EGFR TKD, including this one (PDB entry 2GS6), also show the 986–994 region, including Y992, in a similar location—although not the 971–980 helix.

TOWARD AN UNDERSTANDING OF THE INTACT EGF RECEPTOR

Negative Cooperativity

Although initially satisfying, the basic model presented in Figure 2 for EGFR activation fails to explain several long-standing observations about the receptor—despite the additional complexity added by studies of the JM, TM, and carboxy-terminal tail regions. In particular, as pointed out as the first structures were solved (Burgess et al. 2003), this model fails to capture key qualitative features of EGF binding to the cell surface. As described by Schlessinger (2014), EGFR and the insulin receptor were the first RTKs for which cell-surface ligand binding was analyzed in detail, in the 1970s. Concave-up Scatchard plots were seen in both cases, indicating either heterogeneity of sites or negative cooperativity. In the case of insulin, further experiments showed the binding to be negatively cooperative (De Meyts 2008). For EGFR, in

contrast, heterogeneity of sites was generally assumed, and a great deal of effort was put into understanding the difference(s) between the high- and low-affinity EGF-binding sites (Wofsy et al. 1992; Burgess et al. 2003; Klein et al. 2004)—with the former considered to be the site relevant for cell signaling. More recently, Pike and colleagues (Macdonald and Pike 2008) provided convincing evidence for negative cooperativity in EGF binding to its receptor from experiments in which they globally fit binding data obtained using cells expressing EGFR at a range of different levels. Negative cooperativity (and the characteristic concave-up Scatchards) is seen only for the intact receptor. Deletion of the cytoplasmic domain from EGFR appears to abolish negative cooperativity (Livneh et al. 1986), and further analysis has placed particular emphasis on interactions that involve the iJM region (Macdonald-Obermann and Pike 2009). As is the case with the insulin receptor (De Meyts 2008), negative cooperativity is not observed in

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studies of the soluble extracellular region of the human EGF receptor. The EGFR extracellular region forms a 1:1 complex with EGF ($K_D \sim 400$ nM) that subsequently dimerizes (with $K_D \sim 3$ μ M) to form a 2:2 EGF:EGFR complex (Lemmon et al. 1997) with no significant cooperativity. Interactions involving the intracellular and iJM regions are clearly required for occupation of one binding site in an EGFR dimer to influence binding to the other in negative cooperativity.

Studies of the *D. melanogaster* EGFR (dEGFR) have provided a structural view of how negative cooperativity can arise in ligand binding to a member of the ErbB family (Alvarado et al. 2010). Unlike its human counterpart, dEGFR retains negative cooperativity in ligand binding when the soluble (overexpressed) extracellular region is studied. This protein can also be crystallized as an asymmetric singly ligated extracellular dimer that suggests a straightforward explanation for half-of-the-sites negative cooperativity (Levitzki et al. 1971), as summarized in Figure 5. Intriguingly, dEGFR crystallizes as a dimer even when not bound to ligand (Alvarado et al. 2009), providing a potential structural model for the preformed dimers of EGFR that have been reported in many studies (discussed in Valley et al. 2014 and Arndt-Jovin et al. 2014). This dimer also forms in solution (albeit weakly) and is mediated by the dimerization arm mentioned above. The scheme shown in Figure 5 is based on actual crystal structures. In the absence of ligand, all binding sites are equivalent (as monomers or in symmetric preformed dimers). Upon binding to the left-hand molecule in Figure 5, the Spitz ligand (cyan) “wedges” itself between domains I and III and forces them apart. As a result, domain II (which connects domains I and III) becomes bent and is forced up against domain II from the right-hand molecule, with which it makes a large number of new interactions. The extent of the dimer interface is thus increased substantially in the asymmetric dimer seen in the middle of Figure 5—consistent with the observed ligand-induced dimerization (Alvarado et al. 2010). Importantly, the new asymmetric dimer interface imposes restraints on the

right-hand binding site of the dimer. When a second ligand binds to the dimer, it cannot push domains I and III apart without compromising the asymmetric dimer interface. Indeed, asymmetry is retained in a 2:2 ligand:receptor complex (rightmost structure in Fig. 5), and the second ligand forms a compromised set of interactions consistent with its lower affinity. Thus, binding of the first ligand to a symmetric dimer imposes an asymmetry that reduces the binding affinity of the second ligand in an example of classic half-of-the-sites negative cooperativity (Levitzki et al. 1971; Alvarado et al. 2010).

Negative cooperativity provides a potential mechanism for graded activation of EGFR in response to a concentration gradient of activating ligand (as morphogen), known to be important in several contexts for Spitz and EGFR in *D. melanogaster* (Golembo et al. 1996). It is also likely to be relevant in mammals. Although a singly ligated asymmetric dimer has not been seen for a mammalian ErbB receptor extracellular region, detailed comparison of crystal structures of ligand-bound EGFR and ErbB4 extracellular dimers (Liu et al. 2012) provides compelling arguments that the model shown in Figure 5 is also relevant for human receptors. Moreover, by coexpressing mutated ErbB receptors with defects in ligand binding and kinase activity, respectively, the Leahy laboratory has provided evidence that singly ligated dimers of EGFR and ErbB4 are competent to signal (Liu et al. 2012).

Linkage between Extracellular and Intracellular Regions

As described above, there is now clear evidence for formation of asymmetric dimers in both the extracellular and intracellular regions of EGFR, raising the question of how the two are linked. It would seem reasonable to argue that asymmetry in the extracellular region might promote asymmetry in the intracellular region in a well-defined way, suggesting a view of intact ErbB receptors as allosterically regulated dimeric enzymes with a dimer interface that happens to span the membrane (Lemmon 2009). Experi-

Figure 5. Structural basis for negative cooperativity in an EGF receptor. Negatively cooperative ligand binding is retained by the isolated extracellular region of the *D. melanogaster* EGFR, which binds to the EGF homolog Spitz (Spi), whereas it is lost in studies of the human receptor. Crystallographic studies have revealed the structural basis for this negative cooperativity. The *D. melanogaster* EGFR extracellular region does not form the tethered structure depicted in Figure 2A, remaining as an untethered monomer or forming a symmetric (crystallographic) dimer even in the absence of bound ligand (Alvarado et al. 2009). After binding of ligand to the left-hand molecule in the symmetric dimer, domains I and III are wedged apart as described in the text so that domain II of the left-hand molecule “collapses” onto its counterpart in the right-hand molecule. The result is a singly ligated asymmetric dimer. This transition restrains the second ligand-binding site so that the wedging apart of domains I and III is disfavored, reducing the affinity of ligand for the second site—resulting in negative cooperativity. The structures in the *upper* panel are actual crystal structures (PDB entries 3I2T, 3LTG, and 3LTF) and are redrawn as cartoons in the *lower* part of the figure for clarity (Alvarado et al. 2009, 2010).

mental studies with intact receptors, however, give quite different impressions. As mentioned above, cysteine cross-linking studies in the Springer laboratory (Lu et al. 2010) suggested that the extracellular and intracellular regions of EGFR in cell membranes are only loosely linked. Negative-stain electron microscopy studies of nearly intact EGFR provide a similar impression, suggesting that even with a single dimeric configuration of the EGFR extracellular region, the intracellular region of the same molecule is free to adopt a range of different confor-

mations (Mi et al. 2011). Moreover, in studying signaling by singly ligated EGFR and ErbB4 dimers, Liu et al. (2012) reported that the part of “activator” in the intracellular asymmetric TKD dimer could be played by either the ligand-bound or the ligand-free receptor—again suggesting flexibility or plasticity. In contrast, other studies using mutated receptors have suggested that the extracellular asymmetry in an EGFR/ErbB2 heterodimer is strictly maintained in the intracellular region (Macdonald-Obermann et al. 2012)—with the EGFR TKD

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obligately adopting the receiver position and being activated first.

A flexible linkage between the extra- and intracellular regions of EGFR family members would certainly be sufficient for a signaling mechanism involving straightforward ligand-induced receptor dimerization. It is difficult, however, to understand how preformed receptor dimers could be regulated by ligand binding (Chung et al. 2010) without rigid coupling of extra- and intracellular conformations. Moreover, it seems unlikely that intracellular mutations or deletions in EGFR (or T654 phosphorylation) would be able to influence the cooperativity of extracellular ligand binding (Macdonald-Obermann and Pike 2009) if intra/extracellular linkage is loose. In addition, it is not clear how the different ligands that bind to a given ErbB family member could induce the distinct signaling responses that have been reported (Wilson et al. 2009) if the extra- and intracellular regions were flexibly linked—and there would be no basis for expecting distinct signaling by singly and doubly ligated receptor dimers. Interpreting studies of purified intact ErbB receptors is made difficult by the need to recapitulate the correct membrane environment—including acidic phospholipids and possibly gangliosides—and its asymmetry in order to be sure that the extracellular and intracellular (and, more importantly, the JM) regions are placed in physiologically relevant contexts. Cellular studies are inherently less detailed and quantitative, and—for negatively cooperative systems—it is difficult to be certain that a single receptor conformation is being studied (rather than a mixture of singly and doubly ligated dimers, for example). As discussed elsewhere, however, technologies for studying intact receptors in cells are improving greatly and will soon be at the stage where cellular and structural studies can be productively linked.

CONCLUDING REMARKS

Once considered prototypical RTKs, it is now clear that the EGFR and its relatives are actually quite unique in their properties. Our understanding of their regulation is now quite sophis-

ticated, with multiple structures of extracellular regions and TKDs, and a growing knowledge of the JM and TM regions. Mechanistic studies have provided clear explanations for how oncogenic mutations in the TKD activate EGFR in non-small-cell lung cancer and have aided development of next-generation kinase inhibitors to combat resistance to existing agents. Advances in our understanding of extracellular regulation also promise to explain EGFR mutations in glioblastoma in the future (Lee et al. 2006a; Vivanco et al. 2012). EGFR family members are the only RTKs with a TKD that are regulated without requiring activation-loop phosphorylation—instead using a unique mode of allosteric regulation through asymmetric dimerization. EGFR is also the only well-understood RTK in which bound ligand does not contribute directly to the dimer interface—instead controlling receptor-mediated dimerization. The EGFR family shares more similarities with the insulin receptor than with most other RTKs—including negative cooperativity in its ligand binding, which may reflect its function as a detector of ligand concentration in morphogen gradients. One of the next frontiers in understanding EGFR family signaling will be detailed studies of intact receptors—so that the JM regions can be studied in physiologically (and structurally) relevant environments. Another will be thorough investigation of how the flexible carboxy-terminal tail contributes to EGFR regulation and coupling to downstream signaling. Given the plethora of ErbB family ligands and the wide range of processes in which ErbB receptors participate, it will also be important to relate signaling outcomes to specific ligands (or concentrations of ligands) to unravel the complicated biology of these receptors.

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