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Novel Insights into the Allosteric Activation of the Epidermal Growth Factor Receptor

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Abstract

EGF receptor activation requires both ligand-binding and receptor-mediated dimerization through receptor domain II. The relationship between these processes, however, remains unclear. We have decoupled these processes to examine the ligand-binding affinity and the structure of constitutively-monomeric and -dimeric forms of the EGF receptor, as well as EGF receptor that dimerizes upon ligand-binding. Surprisingly, monomeric receptor binds to the ligands EGF and TGFα with an affinity equivalent to that of dimerizing receptor but with a unique binding enthalpy. This shows that monomeric, ligated EGF receptor adopts a state that is distinct from that of EGF receptor within a homodimer, and this state may be relevant to heterodimeric ErbB signaling complexes. Constitutively-dimerized receptor binds ligand with elevated affinity; however, it still requires ligand to form the receptor domain II dimeric interface. In the absence of ligand, no ordered, receptor domain II-mediated dimer interface is formed. Thus, the affinity effect does not arise from any pre-organization or stabilization of the ligand-binding sites on the receptor, but rather through an entropic effect of enforcing dimerization. Thus, pre-formed human receptor dimers require allosteric activation by ligand in order to signal, and this allosteric mechanism is distinct from that we recently observed for the D. melanogaster EGF receptor. Our observations on the allosteric mechanism of EGF receptor activation prompted us to ask whether other EGF receptor ligands may exert unique allosteric effects. To this end, we investigated the allosteric effects of the ligands Amphiregulin, Epiregulin, and Epigen on EGF receptor. We report that Epiregulin and Epigen, in particular, exert unique allosteric regulation on the receptor, as evidenced by divergent effects of EGFR variants on ligand-binding. Finally, we have studied ligand-binding and dimerization of receptors bearing activating extracellular mutations that cause glioblastoma. We report that these mutations elevate ligand-binding affinity, but they do not drive receptor dimerization. Our findings inform a revised model of ligand-induced receptor activation, in which the dimerization interface is highly sensitive to the presence and the identity of the bound ligand, and the domain I/domain II interface plays a crucial auto-inhibitory role.

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Nicholas J. Bessman

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Chapter 1: EGF receptor signaling, from organismal biology to atomic resolution

Receptor tyrosine kinases: a conserved transmembrane signaling module

Receptor tyrosine kinases, or RTKs, are a conserved family of transmembrane signaling proteins in metazoan organisms. RTKs play a central role in organismal development and patterning. Beyond development, RTKs serve to maintain physiological homeostasis in mature organisms. Many of the earliest biochemical paradigms in physiology and development were, many decades later, found to depend directly upon the transmembrane signaling properties of RTKs. For example, insulin was initially purified in the early 1920s by Frederick Banting and colleagues, and it was immediately shown to cure diabetes when injected at regular intervals(Banting, 1922). Later, Rita Levi-Montalcini and Stanley Cohen, working in close proximity in the 1950s, discovered two molecules with remarkable stimulatory effects on nerve cells and mouse development, respectively(Cohen, 1962; Levi-Montalcini, 1960). These two molecules were termed 'growth factors'. With the advent of recombinant DNA technology and gene cloning in the 1970s and 1980s, it was recognized that the receptors mediating the biological effects of insulin and other growth factors constitute just a handful of examples from a broad and conserved family of RTKs. Each RTK senses a unique set of extracellular ligand molecules and transmits a signal to the interior of the cell by a broadly conserved enzymatic mechanism (Fig. 1). Accordingly, RTKs broadly allow individual cells within a multi-cellular organism to respond appropriately to diverse developmental, physiological, and environmental cues, by exploiting a modular molecular- and network-architecture.
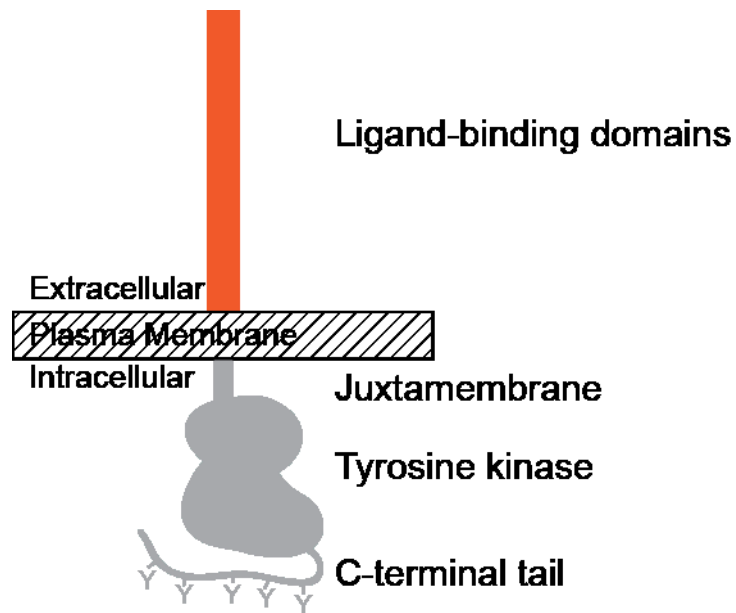
Figure 1: RTKs share a common architecture. In the extracellular regions (orange), a wide variety of protein domains are present. Ligand binds to these extracellular regions. Each RTK sub-family binds a defined set of ligands, defined by the receptor extracellular regions. A single-pass, α-helical transmembrane domain connects the extracellular regions to a short juxtamembrane region, followed by a tyrosine kinase domain. A variable-length, unstructured C-tail region, rich in substrate tyrosines, lies at the C-terminus of the receptor protein.

Biological roles

Because the diversity of the RTK family resides primarily in the extracellular ligand-sensing machinery, RTKs are divided into sub-families based on these extracellular regions(Lemmon & Schlessinger, 2010). Within each human RTK sub-family, there may be as few as one family member, or as many as fourteen. In total, humans express 58 different RTKs, divided into 20 sub-families. Recent bioinformatic analyses have suggested an even broader family of RTKs is expressed in unicellular organisms, but the relevance of these to metazoan RTKs is unclear (Miller, 2012). For the different metazoan species, the number of family members within each

sub-family correlates roughly with the complexity of the body plan and organism size. For example, C. elegans and D. melanogaster express only one ErbB sub-family receptor, whereas mammals express four(Stein & Staros, 2006). Similarly, humans express four fibroblast growth factor (FGF) receptors and 22 different FGF ligand molecules, where C. elegans expresses just two FGF ligands and one FGF receptor(Itoh, 2007). In light of this observation, it is not surprising that RTKs seem to function primarily as master regulators of tissue development.

Each RTK sub-family is generally associated with the development of a particular subset of tissues or cell types. Diversity within RTK sub-families adds another layer of regulation and complexity. In the well-studied insulin receptor family of RTKs, genetic ablation of the signaling pathway universally results in growth retardation, although the magnitude and mechanism of the effect depends on the specific receptor or ligand targeted. In mammals, knockout of the IGF-1 growth factor (which stimulates the IGF-1 receptor) results in decreased bone cell proliferation and a severe growth retardation, whereas insulin receptor (IR) knockout results in a mild growth retardation, due to a slight decrease in adipose tissue mass – with no effect on early bone development(Nakae et al, 2001). Similarly, within the mammalian PDGF receptor family, receptor deletion results in severe developmental defects and embryonic or early post-natal death due to insufficient numbers of specific smooth muscle cell populations in the vasculature. Mice null for PDGF-A (a PDGFRα ligand) lack a population of smooth muscle cells that normally participates in formation of lung alveoli, and these mice display an emphysema-like phenotype at birth. For PDGF-B (a PDGFRβ ligand), knockout results in embryonic death due to massive hemorrhaging(Betsholtz, 2004). Hemorrhaging occurs because PDGF-B is required for the expansion of a vascular smooth muscle cell population that forms the primary wall structure of blood vessels. In yet another illustration of this theme, Trk receptor family members are required for development of different neural tissues; TrkA for nociceptive sensory neurons (TrkA-null mice are insensitive to pain), and TrkB for the vestibular ganglion (TrkB-null mice exhibit defects in balance and locomotion)(Tessarollo, 1998). It is clear that RTKs play a nearly ubiquitous role in

cellular differentiation and proliferation events underlying development, following a prototypical axis in which ligand and receptor act as an autonomous, modular signaling unit.

In contrast to the well-defined and autonomous pathways described above, it must be noted that several RTKs require other accessory proteins, co-receptors, or higher-order oligomerization (beyond dimerization) in order to transmit their signals. For example, the highly-conserved kinase activity within the intracellular domain of RTKs is severely compromised or absent in 8 out of the 58 human RTKs: Ryk, Ror1, Ror2, ErbB3, CCK4, EphA10, EphB6, and SuRTK106(Mendrola et al, 2013; Shi et al, 2010). For ErbB3 and EphB6 in particular, there is good evidence that these receptors can interact with other autonomous RTKs to modulate signaling, perhaps by activating them allosterically (Citri, 2003; Truitt & Freywald, 2011). Furthermore, accumulating evidence suggests that several RTKs function as Wnt receptors or co-receptors. To date, the RTKs MuSK, CCK4, Ryk, and Ror2 have been implicated in Wnt signaling(Niehrs, 2012). Finally, higher-order oligomerization is known to modulate signaling by the Eph, DDR, and Tie receptors(Lemmon & Schlessinger, 2010; Mihai et al, 2009). Understanding RTK signaling through non-autonomous pathways remains a highly active field of research, and these alternative signaling modes remain poorly understood.

Roles in cancer and other diseases

Given the widespread role of RTKs in driving proliferative processes in development, it is no surprise that anomalous regulation of RTKs underlies a wide variety of disease states. Where loss-of-function mutations underlie diverse developmental and degenerative pathologies, gain-of-function mutations are largely associated with cancer development and progression (owing to unchecked cell proliferation and survival). Indeed, the ErbB and Met RTK sub-families (among others) were initially discovered as homologs of viral oncogenes(Dean et al, 1985; Sergeant et al, 1982). The important role of RTK signaling in cancer is reflected by the widespread development and clinical use of RTK inhibitors for cancer therapy. To date, of the 20 RTK sub-families, only 5 are not known targets of cancer therapy development: Ryk, MuSK, CCK4, LMR, and SuRTK106.

The majority of these 'non-targets' are kinase-compromised (Ror, Ryk, CCK4, and SuRTK106), and the remainder (MuSK and LMR) are not known to signal through autonomous ligand-receptor complexes(Mendrola et al, 2013). Among the 14 RTK sub-families targeted in cancer therapy, 11 are targeted by clinically-approved inhibitors, while the remaining three families (Trk, Axl, and Insulin receptor family) are targets of active clinical development(Alamgeer et al, 2013; D'Arcangelo et al, 2013; Festino et al, 2013; Haisa, 2013; Heldin, 2013; Hynes, 2009; Montero et al, 2011; Norris et al, 2011; Paccez et al, 2014; Scagliotti et al, 2013).

Two major challenges for therapeutic RTK inhibition are well-known: specific targeting of RTK kinase activity is difficult to achieve due to the relative conservation across the human kinome(Festino et al, 2013; Gao et al, 2013), and resistance to RTK-directed therapies typically develops on a timescale of weeks or months(Hynes, 2009; Rosenzweig, 2012). Most RTK-directed small molecule therapeutics inhibit multiple kinases. A broad range of kinase inhibition may actually be useful in many disease contexts, but it severely complicates the drug development process. For example, off-target kinase inhibition may underlie undesirable side effects that limit dosing below a crucial therapeutic threshold. RTK-directed therapeutic antibodies sidestep the kinase specificity problem by targeting the diverse extracellular regions of receptors with exquisite specificity. Unfortunately, however, both therapeutic antibodies and small molecules directed at RTKs rapidly lose efficacy in treated patients. The acquisition of specific, recurrent resistance mutations is a rapidly developing paradigm in cancer therapy(Rosenzweig, 2012). One promising approach to combat resistance is rational combination therapy, in which drugs targeting a likely resistance pathway are co-administered with a drug targeting the primary oncogenic driver(Kwong & Davies, 2014). Additionally, expanding our mechanistic understanding of RTK signaling should allow us to more potently and specifically target aberrant RTK signaling in cancer and other diseases.

Whereas cancer therapeutics are designed to inhibit diverse RTK signaling pathways, there is a good biological rationale to develop therapeutic agonists of RTK signaling in other disease

contexts. The classic example, of course, is insulin therapy for diabetes. Beyond insulin, Tie1 receptor stimulation has been investigated as a mechanism of supporting vascular integrity in sepsis(David et al, 2013). Therapeutic potential for HGF, the ligand for Met, has been revealed in both a topical form for recurrent leg ulcers(Nayeri et al, 2002), as well as in gene therapy for treatment of chronic limb ischemia(Shigematsu et al, 2010). Indeed, considering the widespread role of RTKs in vascular development, several RTK ligands have been investigated as therapies for ischemia(Hammer & Steiner, 2013). In addition, great excitement has surrounded the recent discovery that Nrg1, an ErbB ligand, stimulates cardiomyocyte generation in the adult heart and aids recovery from heart failure(Bersell, 2009; Galindo et al, 2013; Hao et al, 2014). The clear therapeutic promise of receptor tyrosine kinase drugs – whether inhibitors or agonists, specific or broad, small molecule or antibody – has prompted detailed mechanistic studies of RTK signaling.

General mechanisms of signaling by receptor tyrosine kinases

At the most general level, RTKs act as transmembrane receptors that convey an extracellular signal into the interior of the cell. RTK extracellular regions (herein referred to as ECRs) directly bind to a ligand, resulting in increased effective kinase activity within the intracellular tyrosine kinase domain of the receptor (Fig. 2). This is the core process of RTK signaling. The increased effective kinase activity of RTKs contributes to intermolecular receptor *trans*-autophosphorylation, in which one receptor molecule phosphorylates another at defined tyrosine residues. Phosphotyrosines on the receptor are then specifically recognized by interaction modules in other signaling molecules that assemble active signaling complexes upon binding to activated receptor. These modules include Src homology 2 (or SH2) domains and phosphotyrosine binding (or PTB) domains. Activated RTK signaling complexes exploit a handful of well-characterized pathways to broadly modulate gene expression, directly giving rise to the multitude of proliferative or differentiation events triggered by RTKs. All the while, physiological RTK signaling is regulated at many levels. Expression of both receptor and ligand is tightly controlled. Constitutively active protein phosphatases at the cell membrane counteract the basal kinase activity of inactive

7

receptors (Sastry & Elferink, 2011). Upon activation of receptor, down-regulation occurs through

of variety of mechanisms.
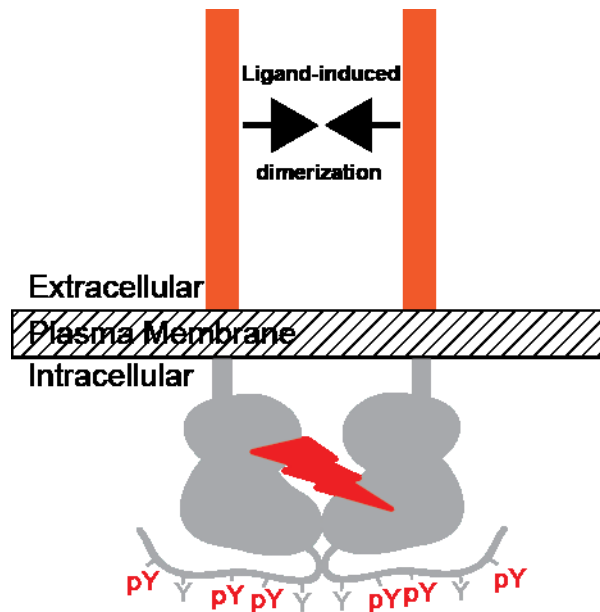
Figure 2: *RTK ligands induce dimerization by diverse structural mechanisms. Upon dimerization, the kinase domains within a dimer become activated. An activated receptor kinase within one protomer proceeds to phosphorylate substrate tyrosines on its dimeric partner.   Receptor phosphorylation generally increases the inherent kinase activity (although ErbBs are an exception to this rule).   Receptor phosphotyrosines serve as docking sites for PTB- and SH2-domain containing proteins, which seed active signaling complexes.*

Ligand-binding and dimerization

For the vast majority of autonomous RTKs, ligand-binding and receptor dimerization are intimately-linked processes that are thought to be tightly coupled energetically. The single well-established exception to this rule is the insulin receptor family, in which receptors are constitutively dimerized via an intermolecular disulfide bond(McKern et al, 2006). In some

contexts, the ErbB family is prone to form ligand-independent dimers as well; this idea will be further examined later, and indeed is a primary focus of this thesis and the active work of others in the field (Chung, 2010; Gadella & Jovin, 1995; Saffarian, 2007; Tao & Maruyama, 2008; Webb, 2008; Yu, 2002). Incidentally, both of these receptor families are now known to utilize a unique structural mode of dimerization, in which the receptor dimer interface is formed exclusively by receptor protein surfaces; the contribution of ligand to dimerization is indirect(Garrett, 2002; Liu et al, 2012a; Ogiso, 2002). At the opposite end of the spectrum, the ligand-dependent, extracellular dimerization interface that activates Trk receptors is exclusively ligand-mediated, with ligand acting as a bivalent, non-covalent cross-linker for two receptor molecules (Banfield et al, 2001; Wiesmann et al, 1999).

Between these two extremes lie all the other structurally-characterized RTKs. For these receptors, the homo-dimeric contacts that stabilize formation of an active dimer are distributed between both receptor-receptor contacts and ligand-ligand contacts, and even ligand-accessory molecule contacts in the case of FGFR, where the FGF ligands bind receptor and heparin concurrently(Ibrahimi et al, 2005). The receptors Kit and VEGFR are two prototypical examples in which ligand-ligand contacts (within a bivalent ligand dimer) as well as receptor-receptor contacts contribute to the stability of active receptor dimers(Brozzo et al, 2012; Leppanen et al, 2013; Yuzawa et al, 2007). The relative topology of ligand-ligand and receptor-receptor contacts in these receptors (as well as PDGFR) is remarkably well-conserved(Yang et al, 2008). The receptor-receptor contacts typically involve membrane-proximal regions of the receptor, whereas the ligand-ligand contacts involve more distal domains of the receptor, away from the membrane. There is some evidence that this sort of dual dimer-interface topology is important in ErbB receptors as well, even though both of the interfaces in this case are completely receptor-mediated(Liu et al, 2012a; Lu et al, 2010; Moriki et al, 2001). Although the relevance of this topology is not completely understood, there is some evidence that the membrane-proximal dimerization interface may play a role in specifying or constraining an activating dimer

relationship, rather than simply increasing dimerization affinity(Brozzo et al, 2012; Yang et al, 2008).

Kinase domain activation and *trans*-autophosphorylation

Just as the ECRs of RTKs are activated through dimerization, so too are the intracellular kinase domains (Fig. 2). Diverse mechanisms underlie the dimerization-dependent kinase activity of RTKs, and there is no general correlation between the respective modes of ECR dimerization and kinase activation within the RTK family. Broadly speaking, kinase domains adopt an array of differently autoinhibited inactive conformations. Receptor dimerization converts these inactive states, by a variety of different mechanisms, into an active kinase conformation that is largely conserved throughout the RTKs(Huse & Kuriyan, 2002). For the ErbB receptor EGFR, dimerization of the kinase domain occurs in an asymmetric 'head-to-tail' arrangement that allosterically activates the kinase domain of one protomer(Zhang, 2006).

For all the other well-characterized RTKs, auto-inhibitory intramolecular interactions suppress kinase activity by sterically blocking the active site; for these kinases, activation requires phosphorylation of key residues to interrupt the auto-inhibitory interaction(Lemmon & Schlessinger, 2010). The residues involved in auto-inhibition may lie in the 'activation-loop' sequence within the kinase domain, the membrane-proximal juxtamembrane region (N-terminal to the kinase domain), or within the disordered tail of the protein, C-terminal to the kinase domain. For these kinases, it's thought that a basal level of kinase activity (even when auto-inhibited) is sufficient to explain the phosphorylation events responsible for reversing auto-inhibition. Within the high local concentration of receptor afforded by dimerization, this low level of activity is sufficient to trigger receptor activation through a first initial *trans*-autophosphorylation step. Following this first trans-autophosphorylation event within a dimer, an ordered and efficient sequence of further trans-autophosphorylation events ensues (Kim et al, 2012).

The number and location of *trans*-autophosphorylation sites varies by receptor. Most are located in the C-terminal tail, the juxtramembrane region, or in the so-called kinase insert domain. Some of these sites serve as direct docking sites for the SH2 and PTB domains of signaling molecules including PI(3)K, Shc, and Grb2(Schulze et al, 2005). Other sites may serve as docking sites for receptor-specific 'substrate' proteins that function as scaffolding proteins; for these receptors, these substrate proteins are required for the full complement of signal transduction. Such substrate proteins include the insulin receptor substrate (IRS) proteins, and the FGF receptor substrate (FRS) proteins. There is emerging evidence that ligand identity and co-receptor engagement may modify the receptor phosphorylation pattern in a way that directs or modulates aspects of downstream signaling(Hartman et al, 2013; Wilson et al, 2012a). Inevitably, RTKs directly stimulate signaling via the ERK and AKT pathways to regulate gene expression patterns. RTK modulation of STAT transcription factors has also been reported, but the mechanistic onnection is less clear and may be indirect(Gao et al, 2007).

Exogenous regulation of RTK signaling activity

A host of accessory factors in the cell serves both to suppress basal RTK signaling activity in the absence of ligand stimulation, and to down-regulate or terminate RTK signaling activity on a timescale of minutes. Two well-known systems largely fulfill these roles. One is the protein tyrosine phosphatase family (PTPs), which exhibit constitutive activity at the cell membrane to convert spurious phosphotyrosines back to benign tyrosine residues and to reverse the effects of ligand activation (Sastry & Elferink, 2011). In addition, activated receptors are internalized by endocytic pathways, and are either degraded in the lysosome or de-activated by exposure to low pH in early endosomes before de-phosphorylation by PTPs and subsequent recycling to the plasma membrane.

The PTP1B protein is a well-established suppressor of basal signaling for EGFR, Met, PDGFR, and IGF1R(Sastry & Elferink, 2011). It has long been appreciated that general PTP inhibition (for example by orthovanadate or H_2O_2) leads to the rapid activation of RTKs, and more recent,

targeted studies suggest that PTP1B is primarily responsible for this effect (Elchebly et al, 1999; Tran et al, 2003). PTPs in general, typified by PTP1B, seem to exhibit constitutive activity with very broad specificity(Stuible & Tremblay, 2010). Given this apparent lack of PTP regulation, it should not be surprising that PTPs play a very complex and convoluted role in vivo. In fact, it is clear that PTP1B is required for transforming activity in some contexts, so it is not simply a global inhibitor of signaling, but may have signal-promoting properties as well(Bentires-Alj & Neel, 2007). Another PTP, known as SHP2 or PTPN11, possesses two phosphotyrosine-binding SH2 domains, which confer the ability to bind directly to RTK signaling complexes via direct interaction with the Gab adaptor proteins; however, SHP2 is not known to play any inhibitory role in RTK signaling, and instead seems to promote a variety of downstream signaling pathways(Sastry & Elferink, 2011). Finally, the PTPN12 protein has also been shown to down-regulate RTK signaling pathways via its phosphatase activity, but it is not known whether it acts primarily on downstream signaling effectors or directly at the RTK(Charest et al, 1997).

Once an RTK has been activated by ligand-induced *trans*-autophosphorylation, the receptor must be inactivated on an appropriate timescale to avoid perpetual signaling. This inactivation is initiated primarily by the endocytosis. Activated receptors are shuttled to early endosomes. In some cases this results in ligand dissociation (due to low pH) and subsequent recycling of de-activated receptor to the plasma membrane. In other cases, low pH does not cause ligand dissocation, and the receptor is sorted to lysosomes where the receptor is proteolyzed and degraded. Many different mechanisms have been implicated in activity-dependent RTK endocytosis, but the primary route under normal physiological conditions is though to depend largely on clathrin-mediated endocytosis (CME)(Goh & Sorkin, 2013). Every major RTK family is known to become ubiquitinated, but the role of direct ubiquitination in CME is receptor-dependent. For example, whereas EGFR and FGFR-2 endocytosis do not depend on receptor ubiquitination, IGF1R endocytosis requires it(Mao et al, 2011). When the concentration of activated receptor at the cell surface reaches extremely high levels (as in the case of cancer cells overexpressing receptor), the ubiquitin-dependent CME pathway becomes saturated, and other endocytic

pathways become relevant, although little is known about the relevance of these pathways in normal physiological conditions (Wiley, 1988).

Biology of ErbB family RTKs

Receptor knockout studies elucidate roles in cardiac and neural development

Individual genetic deletion of the four mammalian ErbB receptors in mice demonstrates that all of these receptors play important roles in normal cardiac and neural development. Global knockout of ErbB2 or ErbB4 results in embryonic lethality at day E10.5 due to defective cardiac trabeculae formation, a process that depends on myocyte proliferation. ErbB4 deletion also compromises development of hindbrain tissue, whereas ErbB2 deletion affects development of cranial neural crest-derived sensory ganglia(Gassmann, 1995; Lee, 1995). Knockout of ErbB3 causes embryonic lethality at day E13.5, with pups exhibiting blood reflux through defective valves due to aberrant cardiac cushion formation. Trabeculae formation appeared normal for the ErbB3-null mice, but the hindbrain was once again severely compromised(Erickson, 1997). Compared to the other ErbB receptors, ErbB1/EGFR seems to play a more global role in development. EGFR deletion has strain- or background-dependent effects, resulting in either embryonic death due to placental abruption, or death three weeks after birth due to multiple organ failure, with defects in the skin, kidney, brain, liver, and the gastrointestinal tract(Threadgill et al, 1995). Rescue of the strain-specific placental defect showed that a broad, strain-independent neurodegeneration program occurs soon after birth(Sibilia et al, 1998). EGFR knockout mice also exhibit enlarged cardiac valves(Chen, 2000).

Ligand knockout studies display complex phenotypes

Not surprisingly, genetic deletion of ErbB ligands phenocopies ErbB receptor deletion in certain contexts; however, it is equally clear that some ligands are redundant in certain contexts. There are nine discrete genes encoding a total of 11 distinct ErbB ligands (each Nrg gene has 2 splice isoforms), and each of these genes has been deleted in mice. Among the ErbB ligand knockout

mice, the most striking phenotypes belong to HB-EGF- and Nrg1-null mice. Global knockout of HB-EGF, a ligand for EGFR and ErbB4 that is expressed in cardiomyocytes, causes severe cardiac dysfunction typified by malformed heart valves (analogous to EGFR deletion) and considerable enlargement of the heart(Iwamoto, 2003; Jackson et al, 2003). Although administration of HB-EGF to these mice caused increased phosphorylation of ErbB2 and ErbB4 as well as EGFR, phenotypic comparisons indicated that Nrg1 is most likely to be the crucial ligand for ErbB2-heterodimer signaling in the heart. Genetic deletion of Nrg1 produces mice with defects in formation of trabeculae (as in ErbB2- or ErbB4-null mice) and the cardiac cushion (as in ErbB3-null mice)(Meyer, 1995). Additionally, Nrg1-null mice exhibit defects in cranial ganglia and Schwann cell development, revealing an essential role for Nrg1 in ErbB2, ErbB3, and ErbB4 signaling in the developing brain. Although the low-affinity Nrg1α isoforms constitute the bulk of Nrg1 mRNAs in cardiac endothelial cells, specific knockout of these isoforms does not appear to impair heart development, suggesting that the high-affinity Nrg1β isoforms are primarily responsible for ErbB2-, ErbB3-, and ErbB4-mediated developmental cues in the heart(Cote, 2005; Li, 2002). In addition to their role in cardiac development, ErbB2, ErbB4, and Nrg1 are crucial for adult cardiac function. Dilated cardiomyopathy occurs in mice with a cardiac-specific conditional knockout of ErbB2(Crone, 2002; Ozcelik, 2002) or ErbB4(Garcia-Rivello, 2005); both of these receptors are expressed in adult cardiomyocytes. Cell-based experiments lend further credence to the notion that Nrg1 signaling through ErbB2/ErbB4 complexes plays a unique role in cardiac physiology. Moreover, cardiac toxicity has emerged as one of the most problematic side effects of ErbB2-targeted cancer therapy (Guglin et al, 2008).

Phenotypes for other ErbB ligand-knockout mice are more convoluted. In contrast to the dramatic cardiac effects of Nrg1 deletion, Nrg2 knockout mice are viable, and exhibit growth retardation and inefficient reproduction(Britto et al, 2004). TGFα deletion yields a 'wavy-hair' phenotype, accompanied by abnormal follicle morphology(Luetteke et al, 1999). Individual deletion of amphiregulin (Arg) results in defects in mammary gland development; deletion of EGF alone shows no overt phenotype, but combinatorial deletion of Arg and EGF shows a further

exacerbated mammary defect(Luetteke et al, 1999). Even mice triple-null for EGF, Arg , and TGFα survive to maturity. The final three ErbB ligands, Betacellulin (Btc), Epigen (Epg), and Epiregulin (Erg), have all been deleted in mice, and none of the resulting knockout mice display overt defects in development(Dahlhoff et al, 2013; Jackson et al, 2003; Lee et al, 2004). The striking contrast in phenotypes for receptor deletion compared to ligand deletion (especially for EGFR, compared to its seven cognate ligands) has sparked the view that most of the ErbB ligand functions are covered by several-fold redundancies and constitute a robust developmental signaling sysem.

Beyond development, recent studies have pushed forward our understanding of ErbB functions in mature animals. These studies have primarily agreed with the view that most ligands are redundant. For example, Epg, Erg, and Betacellulin have all been show to stimulate oocyte maturation in vivo(Park et al, 2004). In response to corneal wounds, the ligands TGFα, Arg, and Betacellulin are all upregulated, and each can contribute individually to the wound healing response(Zieske et al, 2000). Another striking hint at the combinatorial nature of ligand function comes from genomic conservation: the genes encoding Epg, Erg, Arg, and Betacellulin are all located adjacent to each other in mammal genomes(Lee et al, 2004). This pattern in consistent both with a recent origin for these ligands via gene duplication, and with common regulation at the chromatin level.

Mechanistic insight into ErbB signaling

Signaling by the ErbB family underlies developmental processes in metazoans, from D. melanogaster and C. elegans to mammals(Freeman, 1997; Hill, 1992). In adults, ErbB signaling plays a crucial role in homeostasis of the cardiac and reproductive systems as well as wound healing; aberrant ErbB signaling contributes to the development of cancer, heart disease, and neurological disorders. Indeed, genes encoding EGFR and ErbB2 were first identified as oncogenes in the 1980s and have been viewed as important therapeutic targets in cancer ever since(Bublil, 2007). The role of ErbB receptors in cancer, in combination with the discovery of

phosphotyrosine-based signal transduction in the 1980s, has motivated detailed mechanistic

characterization of ErbB receptor-mediated signaling.   The ErbB/EGFR/HER family comprises

four receptors and eleven bona fide ligands in humans. ErbB receptors have a conserved

domain architecture, with the extracellular, ligand-binding region encoding alternating β-

helix/solenoid (domains I and III) and cysteine-rich domains (domains II and IV in humans). The

intracellular region consists of a short juxtamembrane region, a tyrosine kinase domain, and a

regulatory but largely unstructured C-terminal tail. A single transmembrane helix connects the

extracellular region (or ECR) to the intracellular region (or ICR).

Ligand stabilizes active receptor dimers and oligomers

Groundbreaking studies employing cross-linking techniques with antibody-purified EGFR led to

the proposal that EGFR (and, by extension, other ErbBs and RTKs in general) are activated by

ligand-induced dimerization(Yarden & Schlessinger, 1987a; Yarden & Schlessinger, 1987b).

Although a complete mechanistic understanding of ligand-mediated receptor activation is lacking,

high-resolution crystallographic structural models of receptor fragments have greatly informed the

current view (Fig. 3). Crystal structures of all four of the ErbB receptor extracellular regions have

been solved in the absence of ligand(Bouyain, 2005; Cho, 2002; Cho, 2003; Ferguson et al,

2003; Garrett, 2003). The EGFR ECR structure has also been determined in the fully ligand-

occupied state, with either EGF or TGFα as the activating ligand (Garrett, 2002; Lu et al, 2010;

Ogiso, 2002), and a Nrg-activated ErbB4 ECR structure has recently been reported as well(Liu et

al, 2012b). All of the ligand-regulated receptors (EGFR, ErbB3, and ErbB4) exhibit a compact,

'tethered' conformation in the absence of ligand. The tethered conformation is stabilized by an

intramolecular interaction involving a protruding β-hairpin on domain II and a small surface on

domain IV (adjacent to another, smaller, β-hairpin). The tethering interaction comprises 4-5

hydrogen bonds involving conserved residues in domains II and IV. In ErbB2, which exhibits an

extended, untethered conformation in crystals and in solution(Dawson et al, 2007), these

hydrogen-bonding residues are not conserved(Burgess, 2003; Cho, 2003). ErbB2 cannot form

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the autoinhibitory tether, and may therefore remain constantly poised to heterodimerize with other ligand-bound ErbB receptors.
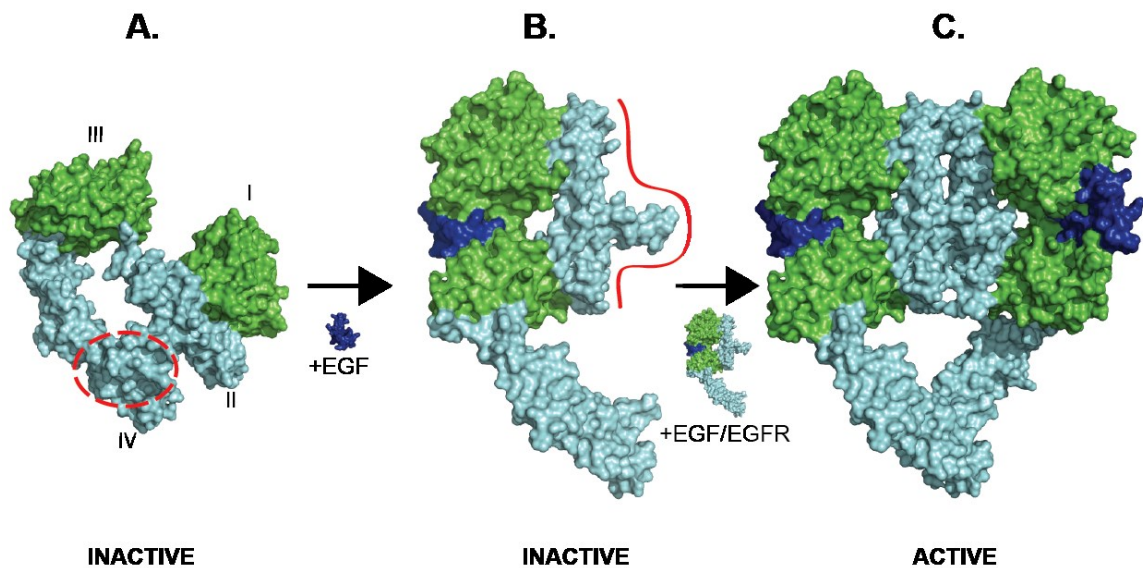
Figure 3: *A model of ligand-induced dimerization and receptor activation based on crystal structures and solution scattering data. A: tethered EGFR (adapted from crystal structure, PDB 1NQL) B: ligated, extended, monomeric EGFR, inferred from low-resolution SAXS data (Dawson et al, 2007) C: ligated dimeric EGFR (adapted from crystal structure, PDB 3NJP).*

The independent structures of ligand-bound (presumably activated) EGFR and ErbB4 ECRs show largely conserved ligand:receptor and receptor:receptor interactions(Garrett, 2002; Liu et al, 2012b; Ogiso, 2002). Ligand binds to surfaces from the β-helix/solenoid domains I and III, and stabilizes a rearrangement of the ECR from a tethered to an 'extended' conformation. The ligand fold is stabilized by three characteristic intramolecular disulfide bonds, though much of its receptor-interaction surface is contributed by an otherwise unstructured C-terminal extension. The extended, ligand-bound receptor is competent for dimerization as illustrated in Fig. 3. It is striking that the dimerization interface is dominated by the β-hairpin 'tether' from domain II, which also stabilizes the inactive conformation. Indeed, mutating the domain II β-hairpin compromises the ligand-regulation of ErbB receptors in cells (Dawson, 2005). An additional contribution to the

dimerization interface is made by residues near the N-terminal part of domain II. Importantly, the EGF- and TGFα-bound EGFR structures differ significantly in this N-terminal part of the domain II dimer interface. The origin and significance of this structural difference is currently unclear; it may arise simply from crystal packing differences, or as a consequence of the different receptor protein boundaries used in the two structures. Alternatively, and as suggested by recent a recent study employing a conformation-specific imaging technique in cells (Scheck et al, 2012), the distinct ligands may inherently specify slightly different dimeric interfaces. This could explain reported ligand-specfic signaling responses (Wilson et al, 2012a). Biophysical studies argue that this portion of the dimer interface makes a small contribution to the overall dimerization energy(Dawson, 2005). However, this does not mean that the structural difference is not biologically relevant. Indeed, membrane-proximal contributions to dimerization of the Kit receptor have been shown to make little or no contribution to dimerization affinity *per se* (Lemmon et al, 1997), but they do make crucial contributions to Kit activation – both by ligand and by oncogenic mutations (Yuzawa et al, 2007).

ECR structures from the ErbB family argue that dimerization underlies ErbB signal transduction (Fig. 3). Structures of intracellular regions argue that formation of an asymmetric dimer of the tyrosine kinase domains, in which one kinase domain allosterically activates the other, allows signal transduction to occur(Zhang, 2006). The juxtamembrane region of the receptor also contributes to the stability of this asymmetric dimer(Red Brewer et al, 2009). However, it is impossible at this time to fully reconcile our understanding of the ligand-mediated extracellular signaling events with the details and intricacies of intracellular kinase domain regulation (Lu et al, 2012; Mi et al, 2011; Moriki et al, 2001; Roberts et al, 2012). Domain IV of the ECR is highly flexible, especially in activated receptor dimers, so the orientation of receptors relative to the membrane remains an open question. It has also been reported, using detergent-solubilized receptors, that the ligand-bound ECR can form an active dimer structure while the inhibitor-bound kinase domains remain monomeric (Lu et al, 2012).

While the structural models described above can inform and explain ligand-induced dimerization, kinase domain activation, and subsequent signal transduction, a wealth of observations suggesting that current structural models are far from sufficient. First, numerous experimental methods have indicated the existence of pre-formed, inactive receptor dimers in the absence of ligand(Gadella & Jovin, 1995; Saffarian, 2007; Webb, 2008; Yu, 2002). This observation undermines the simple model of receptor activation through ligand-induced dimerization. The notion of physiologically-relevant, inactive, ligand-independent receptor dimers is bolstered by recent crystal structures and biochemical studies of the D. melanogaster EGF receptor(Alvarado et al, 2010; Alvarado, 2009). Second, the complex binding characteristics of EGF and other ErbB ligands to cell-surface receptors, first noted 30 years ago, can not be explained based on the available structures(MacDonald, 2008; Ozcan, 2006; Shoyab, 1979). It now appears that the complex ligand-binding characteristics reflect negative cooperativity, as seen in many receptor families (De Meyts, 2008). The origin of this cooperativity – and its modulation by intracellular modifications (MacDonald-Obermann, 2009) – cannot be explained by simple dimerization models, because the crystal structures of human EGFR display symmetric ligand-binding sites. Third, there is evidence that distinct ErbB ligands induce distinct receptor responses at the level of phospho-tyrosine site usage and endocytosis kinetics(Hobbs, 2002; Roepstorff et al, 2009; Wilson et al, 2012a); these data suggest ErbB receptors can discriminate between ligands, which implies that multiple different activated receptor states may coexist.

In combination, these three observations (ligand-independent dimers, negatively-cooperative ligand-binding, and ligand-specific receptor responses) strongly challenge the prevailing structure-based signaling model in which ligand-induced dimerization is the essence of ErbB receptor activation. These challenges argue that subtle, unappreciated modes of allosteric must contribute importantly to the regulation of ErbB receptor activation. The goal of my thesis was to further elucidate how ligand activates the EGF receptor through unappreciated allostery. Success in this regard would yield insight into the activation of ErbB receptors and other RTK

families, and it could also further inform the design of more effective ErbB-targeted therapeutics for cancer and heart disease.

CHAPTER 2: Probing the relationship between ligand-binding and EGF receptor dimerization and activation

Pre-dimerized EGFR requires ligand to form a domain II-mediated dimer

The structural model depicted in Figure 3 implies that EGFR activation is reducible to a ligand-induced dimerization event. In contrast, recent findings from the D. melanogaster system imply that EGFR activation requires ligand-induced allosteric changes within a pre-formed, ligand-independent dimer(Alvarado et al, 2010). Extensive support for each of these models has been reported. These two distinct models for understanding how ligand activates EGFR are not mutually exclusive, but they do have very important implications for therapeutic targeting of EGFR. For example, development of therapeutic antibodies against EGFR has, to date, focused on selecting antibodies that prevent receptor dimerization. If stable, inactive EGFR dimers form in tumors, then antibodies that stabilize such a complex might provide a unique therapeutic effect. While there is evidence that inactive, ligand-independent EGFR dimers are ubiquitous in a cellular context, the isolated human EGFR ECR (utilized for biochemical, biophysical and structural studies) does not form a ligand-independent dimer. To better understand how ligands can activate ligand-independent ('pre-formed' or 'pre-dimerized') EGFR dimers, we genetically fused a dimerizing Fc domain to the EGFR ECR for *in vitro* analyses. This pre-dimerized EGFR, termed sEGFR-Fc, was amenable to thermodynamic, biophysical, and structural studies.

To determine the gross domain arrangement of pre-formed EGFR dimers, we collected and analyzed electron microscopy (EM) images utilizing uranyl formate negative-stain to generate contrast. Class-averaging of single particles obtained from images of the sEGFR-Fc protein in complex with EGF yielded clear densities for all 4 receptor domains as well as the Fc domain and EGF (Fig. 4A). The ligand-receptor complex itself bears a striking resemblance to previously published EM class-averaging results, for both isolated receptor ECRs and for near full-length receptors reconstituted in detergent micelles(Mi et al, 2011). In a subset of classes, the Fc-domain appears well-resolved with good signal, indicating that the particular arrangement of the Fc domain relative to the receptor domains represents a highly-populated state. The relative position of the Fc domain in these classes is reminiscent of the relative position of the kinase

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domains in near full-length receptors. Thus, it is clear that pre-formed human EGFR dimers can

form a domain II-mediated dimer in the presence of EGF.

A

B p = 0.006

Rg, Angstroms

- sEGFR-Fc alone
- sEGFR-Fc + EGF
- Model: Tethered/Splayed
- Model: Tethered/Adjacent
- Model: Extended/DomainII-dimer

C p = 0.02

Dmax, Angstroms

- sEGFR-Fc alone
- sEGFR-Fc + EGF
- Model: Tethered/Splayed
- Model: Tethered/Adjacent
- Model: Extended/DomainII-dimer

D 90°

Model: Extended/DomainII-dimer

E 90°

Model: Tethered/Adjacent

F 90°

Model: Tethered/Splayed
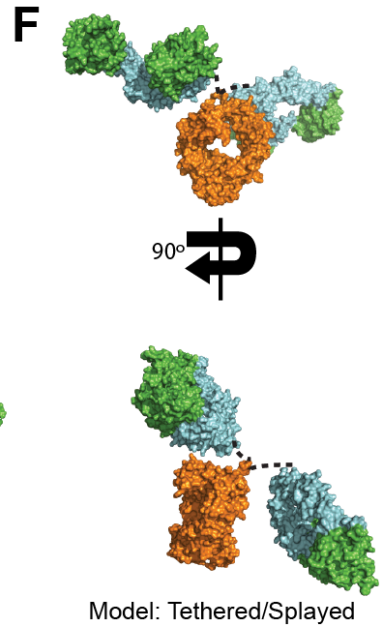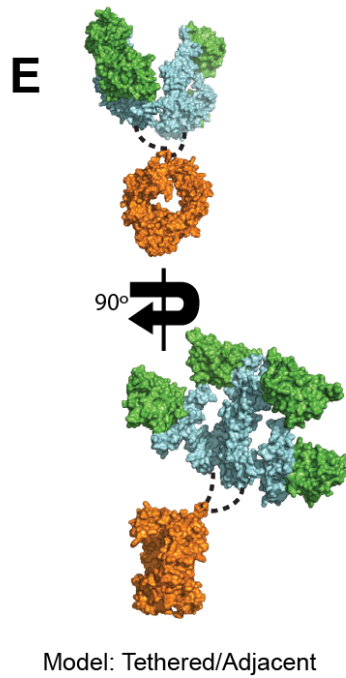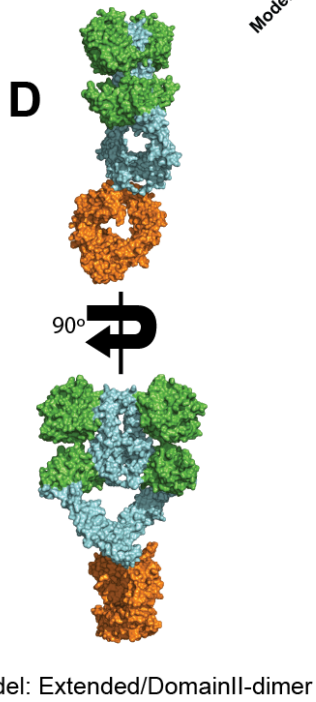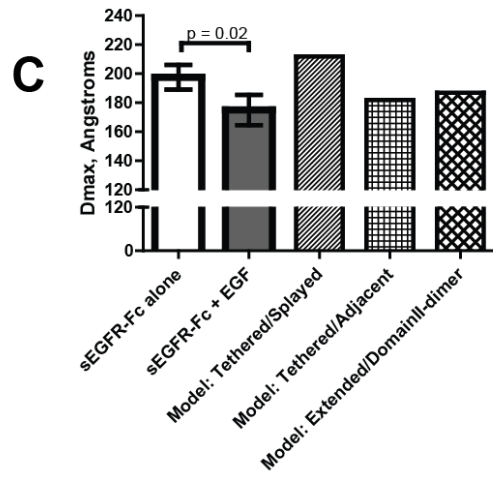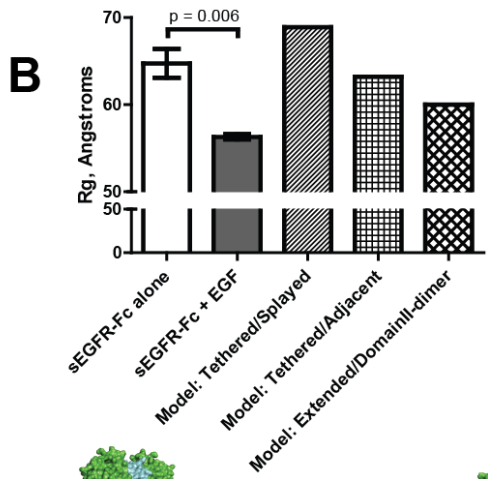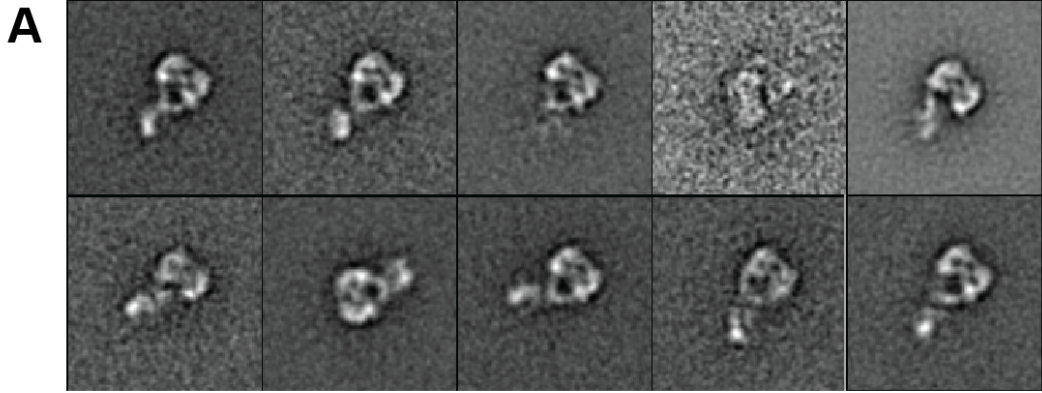
Figure 4: A: Reference-free class averages generated in the Spider software suite, using single-particle EM images of EGF:sEGFR-Fc complexes. B: Rg values derived by Guinier analysis of SAXS data, or calculate from the structural models described in D-F using the HydroPro software. SAXS scattering was recorded for 10-20 µM sEGFR-Fc in the presence or absence of a 1.3-fold excess of EGF. C: Dmax determinations, as previously described. For B.) and C.), four independent experiments were performed, and the mean is plotted with error bars representing the standard error of the mean (SEM). Paired t-tests were used to compare SAXS parameters in the presence or absence of EGF, and the p-values are indicated. D-F: Distinct structural models were constructed; Rg and Dmax values were back-calculated from these models using HydroPro, for comparison to experimentally-determined parameters for sEGFR-Fc.

sEGFR-Fc without added ligand was also imaged by electron microscopy and particles were subjected to the same class-averaging algorithms as used for ligand-bound sEGFR-Fc (Fig. 5); in contrast to the results for the ligand-bound complex, sEGFR-Fc alone failed to generate signal-enhanced class averages with well-defined and interpretable inter-domain relationships (even when using the exact same protein preparations). This result reveals that a well-ordered, back-to-back dimer, mediated by interactions involving domain II of each protomer, does not form in the absence of ligand. It is not clear whether the individual receptor molecules with the Fc-mediated dimer maintain a tethered or extended conformation, or represent an ensemble that samples multiple conformations. These data do argue, however, that forcing dimerization of the EGFR extracellular region (by Fc fusion) does not simply drive it to form domain II-mediated dimers of the sort seen in our crystallographic studies of the *D. melanogaster* EGFR (Alvarado et al, 2010).

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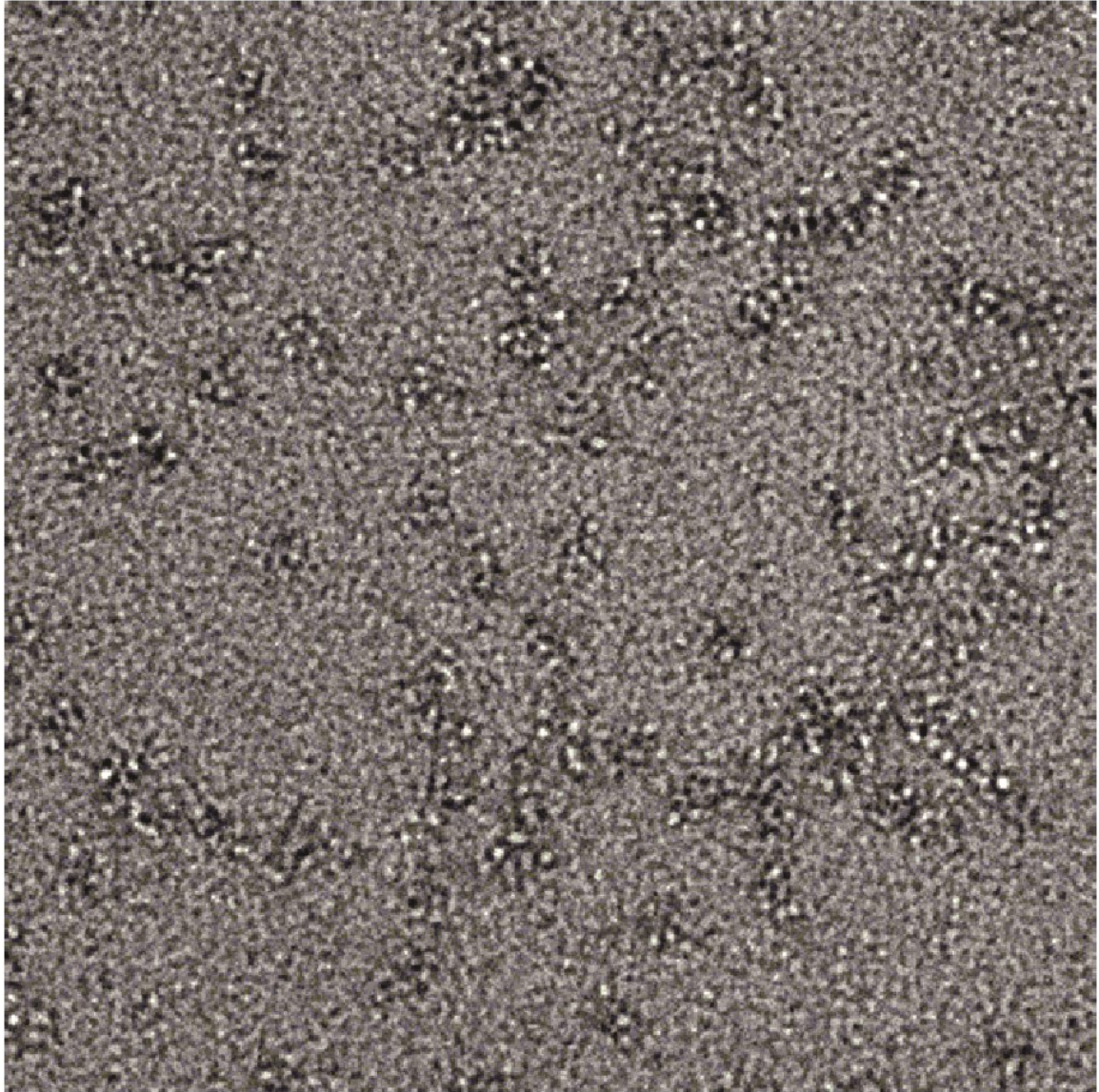
Figure 5: *An example of a raw EM image file (in this case, sEGFR-Fc with no ligand) used for single-particle analysis. For more details, see experimental methods.*

In an effort to better understand the allosteric effect of ligand-binding on pre-formed human sEGFR dimers, we also performed SAXS experiments on sEGFR-Fc in the presence and absence of EGF. For sEGFR-Fc in complex with EGF, we determined the radius of gyration (Rg) by Guinier analysis and the maximal interatomic distance (Dmax) as described

previously(Dawson et al, 2007). In the presence of EGF, the experimentally-determined scattering parameters are in good agreement with the parameters predicted from a structural model in which the previously observed back-to-back extended receptor dimer has an Fc domain dimer appended at the C-terminus of EGFR domain IV, with the Fc dimer extended away from the receptor (Fig. 4B-D). In contrast, the sEGFR-Fc protein alone generates a scattering curve with a significantly larger Rg and Dmax. These scattering parameters are most consistent with structural models in which the two receptor protomers in the unligated sEGFR-Fc dimer are not interacting in an ordered back-to-back dimer (Fig. 4E,F). In agreement with EM data, these SAXS data suggests that sEGFR-Fc – and, by extension, physiological EGFR pre-formed dimers – requires ligand binding in order to form the domain II-mediated dimerization interface seen crystallographically for human and Drosophila EGFR. This conclusion is further supported by recently published EM data on near full-length ligand-independent EGFR dimers – in which dimerization was driven by an EGFR kinase inhibitor rather than an Fc-fusion approach (Lu et al, 2012). In that case, antibody-labeling showed that the extracellular regions of the protein were conformationally heterogeneous. The notion that ligand-independent EGFR dimers require ligand to form a stable, domain II-mediated dimer stands in surprising contrast to the allosteric activation mechanism describes for *D. melanogaster* EGF receptor (Alvarado et al, 2010). Given our surprising findings, we sought to re-examine other key assumptions about dimerization-dependent EGFR regulation, which were originally inferred from the structure-based model presented in Figure 3.

EGFR dimerization does not stabilize ligand-binding

As outlined in Fig. 3, the EGFR ECR monomer adopts a compact, tethered structure in the absence of ligand. The intramolecular tethering interaction sterically blocks receptor dimerization by occluding the domain II dimerization interface. Upon ligand-binding, receptor domains I and II move with respect to domains III and IV such that ligand can bind to surfaces on domains I and III simultaneously(Dawson et al, 2007). This movement (a rigid body rotation about an axis close to

the domain II/III connection) exposes the domain II dimerization arm as shown in the middle panel of Fig. 3, and two 1:1 receptor:ligand complexes then form a symmetric homodimer (Fig. 3C) with a dissociation constant of ~1 μM(Dawson, 2005). This corresponds to a Gibbs free energy (ΔG) of -7.5 kcal/mol for dimerization. Several crystal structures support the views depicted in Figure 3 A and C for both EGFR and ErbB4(Bouyain, 2005; Burgess, 2003; Liu et al, 2012a). The structure of a ligand-bound, monomeric receptor has not been determined at atomic resolution and physiologic pH, but small-angle x-ray scattering (SAXS) data argue strongly that such a complex can adopt an extended, non-tethered conformation (as depicted in Fig. 3B), grossly reminiscent of one half of a 2:2 ligand:EGFR dimer(Dawson et al, 2007).

To examine the energetic linkage of ligand-binding and dimerization for EGFR, we exploited isothermal titration calorimetry (ITC) to compare the thermodynamics of ligand-binding to either wild-type EGFR ECR (sEGFR[WT], residues 1-618 of mature human EGFR) or the Y251A/R285S variant (sEGFR[Y251A/R285S]). sEGFR[Y251A/R285S] does not dimerize upon ligand-binding, and this combination of mutations also abolishes signaling by the full-length receptor (Dawson, 2005; Ogiso, 2002). Titration of the ligand EGF into sEGFR[WT] (Fig. 6A) and into sEGFR[DomainIII] (Fig. 7A) is consistent with previously published data. Without the benefit of dimerization-deficient variants, these earlier studies interpreted wild-type titrations to represent enthalpically-driven ligand-binding and strongly entropically-driven receptor dimerization(Alvarenga et al, 2012; Lemmon, 1997). It is clear, however, from the titration of EGF into the non-dimerizing sEGFR[Y251A/R285S] variant that ligand-binding itself is driven by strongly favorable entropic terms (Fig. 6C). This finding calls for a reinterpretation of previous thermodynamic studies of EGF/EGFR interactions. Based on a calorimetric dissociation experiment (Fig. 7B) the enthalpy of dimerization of ligand-bound EGFR is <<|2| kcal/mol. Because the dimerization enthalpy is insignificant in this context, sEGFR[WT] as well as sEGFR[Y251A/R285S] titrations can be accurately fit with a single-site binding model, where the binding event represents ligand binding to receptor. This thermodynamic pattern – namely, strongly entropically-driven ligand-binding, exaggerated by ~2 kcal/mol for

dimerization-deficient receptor – is maintained for the EGFR ligand TGFα, as well as EGF (Fig. 6, Table 1).

Table 1

EGFR variant	Ligand	$K_D^{\text{Ligand-induced dimerization}}$, μM	$K_D^{\text{Ligand-binding}}$, nM	$\Delta H^{\text{Ligand-binding}}$, kcal/mol
Wild-type	EGF	~1	41 ±12	+6.1 ±0.1
	TGFα	~1	54 ±13	+6.1 ±0.1
Y251A/R285S	EGF	>200	64 ±20	+7.9 ±0.2
	TGFα	>200	65 ±16	+7.6 ±0.3
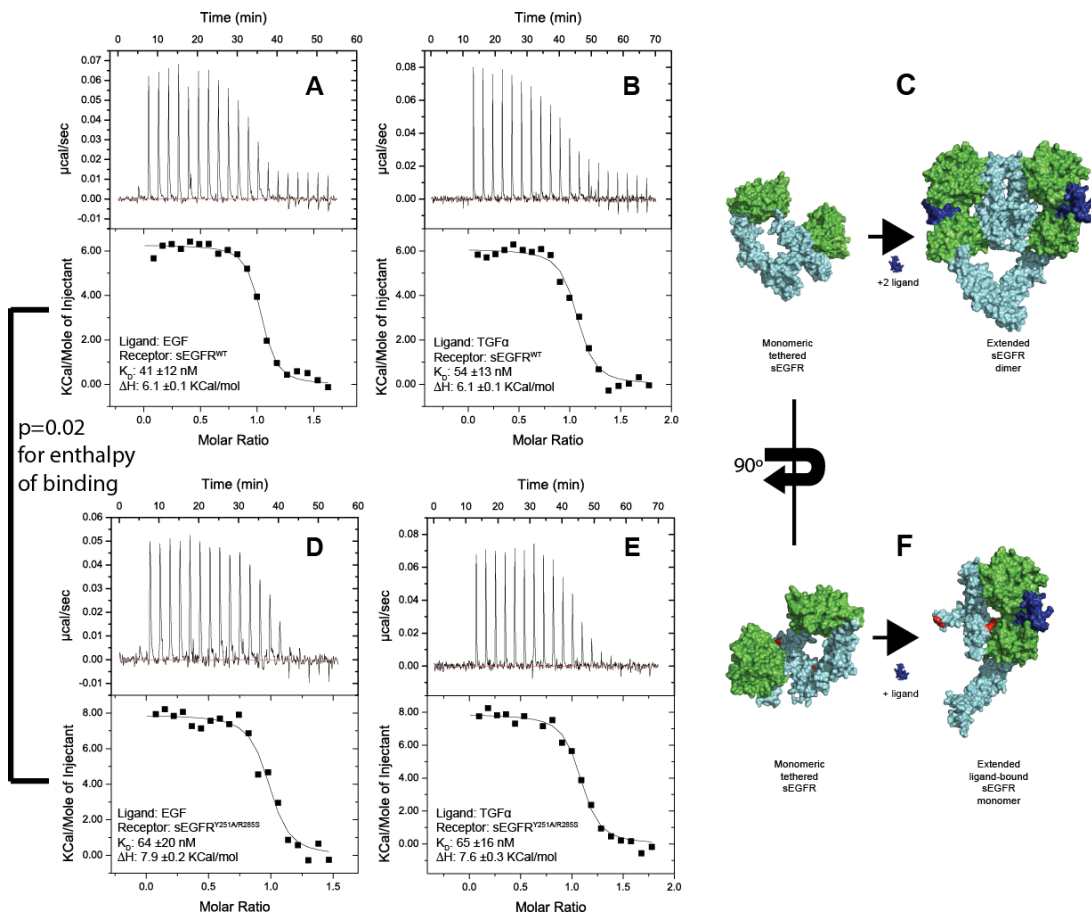
Figure 6: ITC titrations A: EGF titrated into sEGFR^{WT}(5 independent experiments performed; 1 representative titration is shown) B: TGFα titrated into sEGFR^{WT} (2 independent experiments performed; 1 representative titration is shown) C: A structural illustration of the process occurring

during titration of ligand into sEGFR^WT D: EGF titrated into sEGFR^{Y251A/R285S} (3 independent experiments performed; 1 representative titration is shown) E: TGFα titrated into sEGFR^{Y251A/R285S} (2 independent experiments performed; 1 representative titration is shown) F. During ligand titration into sEGFR^{Y251A/R285S}, ligand binds to the receptor and the tether interaction is released, resulting in a relatively extended receptor conformation, but the receptor does not dimerize. Positions Y251 and R285 are highlighted in red. An unpaired student's T-test comparing the ΔH value for EGF binding to sEGFR^WT and sEGFR^{Y251A/R285S} determined a p-value of 0.02, indicating high confidence that ligand-binding to sEGFR^WT and sEGFR^{Y251A/R285S} is qualitatively and significantly different.

Further insight into the relationship between ligand-binding and receptor dimerization can be gained by comparing the overall affinity of EGF for sEGFR^WT and sEGFR^{Y251A/R285S}. If the model in Fig. 3 is accurate, and monomeric, ligated receptor mimics one half of a 2:2 receptor dimer, then the Gibbs free energies of ligand-binding (-9.7 kcal/mol, based on a K_D for ligand-binding of 41nM) and receptor dimerization (-7.5 kcal/mol, based on a K_D for dimerization of ~1μM) should be additive; in other words, receptor dimerization should strongly stabilize ligand-binding, just as ligand-binding stabilizes dimerization in this simple model. In the most extreme case, the K_D for ligand binding to dimerizing receptor should therefore be nearly 6 orders of magnitude lower than for non-dimerizing receptor, corresponding to 7.5 kcal/mol of free energy gained from dimerization. Contrary to this expectation, we find that the ligand-binding affinities for sEGFR^WT and sEGFR^{Y251A/R285S} are equivalent within the sensitivity of the ITC experiment (Table 1). This finding agrees with recent studies of ligand-binding to full-length receptor in cells, in which model fitting of EGF-binding data collected for cells expressing a wide variety of different receptor levels indicated negative-cooperativity, but a similar ligand-binding affinity for monomeric and dimeric receptor (MacDonald, 2008). The notion that monomeric and dimeric receptors bind EGF with equivalent affinities is further supported by comparing the EGF-binding affinity for sEGFR^WT determined by ITC with that determined by a fluorescence anisotropy (FA) assay, as illustrated in Figure 8D. The FA assay employs much lower receptor concentrations, well below the K_D for

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dimerization of ligand-bound sEGFR, so this assay effectively reports on EGF binding to monomeric sEGFR^{WT}. Nonetheless, EGF binds sEGFR^{WT} with an affinity of 78nM in FA assays, arguing that its affinity for sEGFR is not affected significantly by dimerization *per se*. Affinities for EGF binding to wild-type receptor in ITC (where the receptor dimerizes) and in fluorescence anisotropy assays (where sEGFR does not dimerize) are essentially the same, and equivalent to that measured by ITC for the sEGFR$^{Y251A/R285S}$ variant, arguing that receptor dimerization does not stabilize ligand-binding.
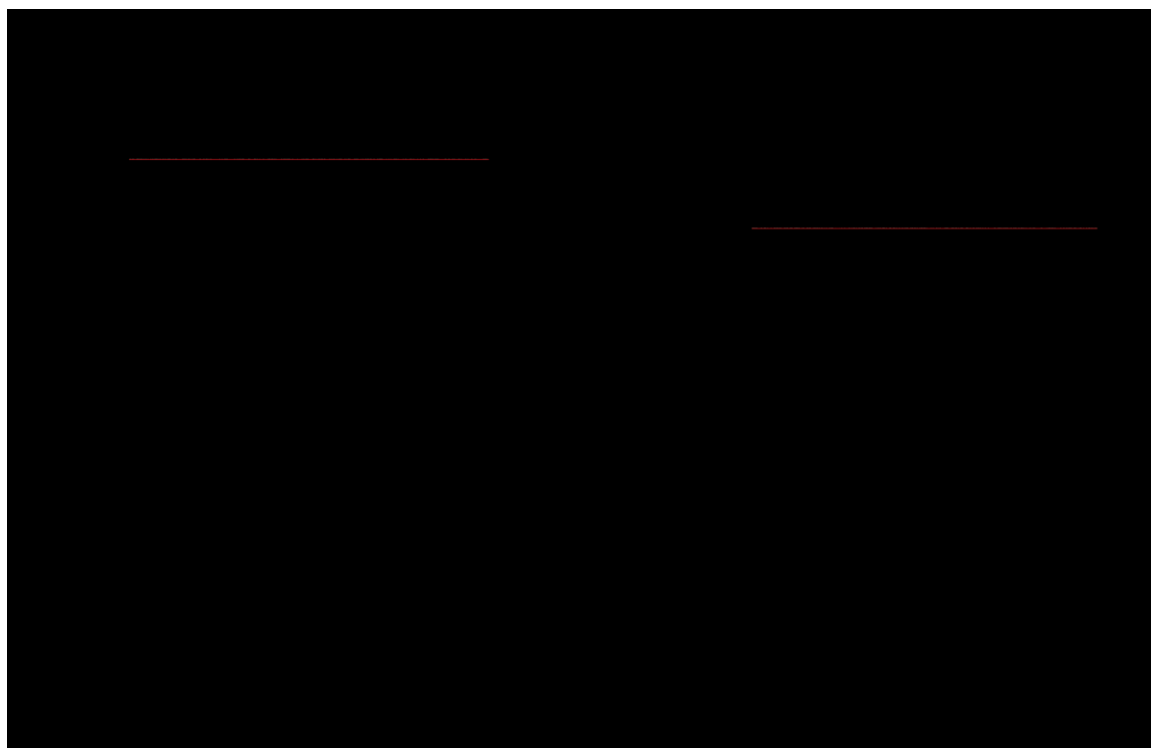
Figure 7: A: ITC titration of EGF into 10µM sEGFR domain III. Fitting to a single binding-site model yielded a K$_D$ of 3.1 ±1.9 µM, and a ∆H of -3.5 ±1.1 kcal/mol. B: To measure the heat of ligand-induced dimerization, 17.5 µM sEGFR^{WT} in complex with a 1.3-fold excess of EGF was injected into an ITC cell containing only buffer. 13 injections of 3 µL were performed, allowing measurement of dissociation heats in the ITC cell over an equilibrium concentration range of ~0.2 – 2.4 µM for the sEGFR^{WT}:EGF complex. Because the heat of injection does not change systematically from the first injection to the last, and because the integrated heat of each injection

is so low (and can not be distinguished from instrumental noise), we conclude that the $\Delta H_{dimerization}$

for the sEGFR^{WT}:EGF complex is <<|2| kcal/mol.

Two independent lines of evidence argue that ligated, monomeric receptor is qualitatively distinct

from one half of a 2:2 EGF:EGFR dimer. If these two species were the same, dimerization of the

two identical halves of the 2:2 EGF:EGFR dimer would lead to their mutual stabilization. In other

words, dimerization would stabilize the ligand-bound conformation, which would in turn be

manifest as an enhancement of ligand binding upon dimerization. As shown above, however, this

does not happen. It seems reasonable to argue instead, from the data outlined above, that

dimerization of a 1:1 EGF:sEGFR complex must compromise ligand binding to each protomer to

some degree. Whereas monomeric, ligated receptor presumably adopts a conformation that

maximizes ligand-receptor interactions, dimerization presumably compromises some of these

interactions, so that ligand-binding and dimerization energies are not additive. In other words, in

order for dimerization to occur, some ligand-receptor contacts must be compromised. We would

therefore expect that the domain II conformation would be different (possibly very subtly) in the

context of a 1:1 EGF:EGFR complex and a 2:2 EGF:EGFR dimer. Second, the enthalpic

signatures for EGF and TGFα binding differ significantly for sEGFR^{WT} and sEGFR$^{Y251A/R285S}$ (Fig.

6 and Table 1). Because dimerization does not contribute significantly to the measured

enthalpies (see Fig. 7B), the difference in ΔH of ~2kcal/mol between sEGFR and the

Y251A/R285S variant can be directly attributed to differences in ligand-binding. The best

explanation for this difference in $\Delta H^{ligand-binding}$ is that the ligand:receptor interaction is qualitatively

(and thermodynamically) distinct for monomeric and dimeric receptor. K_D values are very similar

(64nM and 41nM respectively), so ΔG differs by less than 0.23 kcal/mol. Yet, ΔH for EGFR

binding differs by ~2 kcal/mol, being enthalpically less unfavorable in the case where dimerization

is possible. These observations argue that ΔG for EGF binding to sEGFR dimers remains

essentially the same as that seen for monomers because of a compromise between

enhancements as a result of dimerization and detractions as a result of restraints imposed upon the dimerizing sEGFR molecules.

Regardless of the precise energetic origin of this effect, the equivalence of EGF and TGFα affinity for monomeric and dimerizing EGFR argues that driving EGFR homodimerization is not the primary function of EGF and TGFα – which contradicts the paradigm that has guided mechanistic thinking about EGFR for some time (Lemmon, 1997). This has particularly important implications for understanding ligand activation in cells expressing the modest levels of EGFR typical in physiological conditions. For cells expressing higher levels of EGFR (as in many cancers), however, pre-formed EGFR dimers are an important site of signaling, particularly at low ligand concentrations(Chung, 2010). In this context, the monomeric ligand-bound receptor is less likely to form – raising the question as to how signaling through a pre-formed dimer is achieved.

Pre-dimerized EGFR binds ligand with a high affinity driven by entropy

The data presented above show that the process of dimerization does not increase ligand-binding affinity – with dimerization-defective EGFR binding ligands just as tightly as dimerizing receptor. However, several previous studies have indicated that forcing EGFR into a dimer can elevate its affinity for EGF. Forcing the EGFR ECR into a constitutive dimer by appending a disulfide-linked Fc domain from human IgG1 has been reported to increase ligand-binding affinity relative to wild-type receptor(Adams et al, 2009; Jones, 1999). The presence of ligand-independent receptor dimers in cells, and the intrinsic ability of isolated EGFR kinase and transmembrane (TM) domains to dimerize (Jura, 2009; Mendrola et al, 2002), both argue that pre-formed EGFR dimers are an important species physiologically. To investigate ligand-binding to pre-formed EGFR dimers, we have studied two distinct types of EGFR pre-formed dimers.

To rule out any artifacts attributable to interactions of the Fc domain with receptor or ligand in an Fc-fusion, we studied not only a C-terminally-fused EGFR-Fc pre-formed dimer ('sEGFR-Fc'), but also a C-terminal fusion of the leucine zipper from the *S. cerevisiae* transcription factor GCN4 to the EGFR ECR ('sEGFR-Zip'). A fluorescence-anisotropy (FA)-based experiment shows that

both the Fc-fused sEGFR and the zipper-fused sEGFR bind EGF with an affinity ~10-fold higher

than wild-type (Fig 8D; Table 2). The fact that sEGFR-Zip and sEGFR-Fc both bind EGFR with

very similar K_D values argues that the increase in ligand-binding affinity is a direct consequence

of enforcing dimerization at the ECR C-terminus (equivalent to the region normally connected to

the N-terminus of the TM domain in full-length receptor) and is not an artifactual consequence of

any specific structural mode of dimerization, or of interactions between the dimerizing domain and

either EGF receptor or ligand.
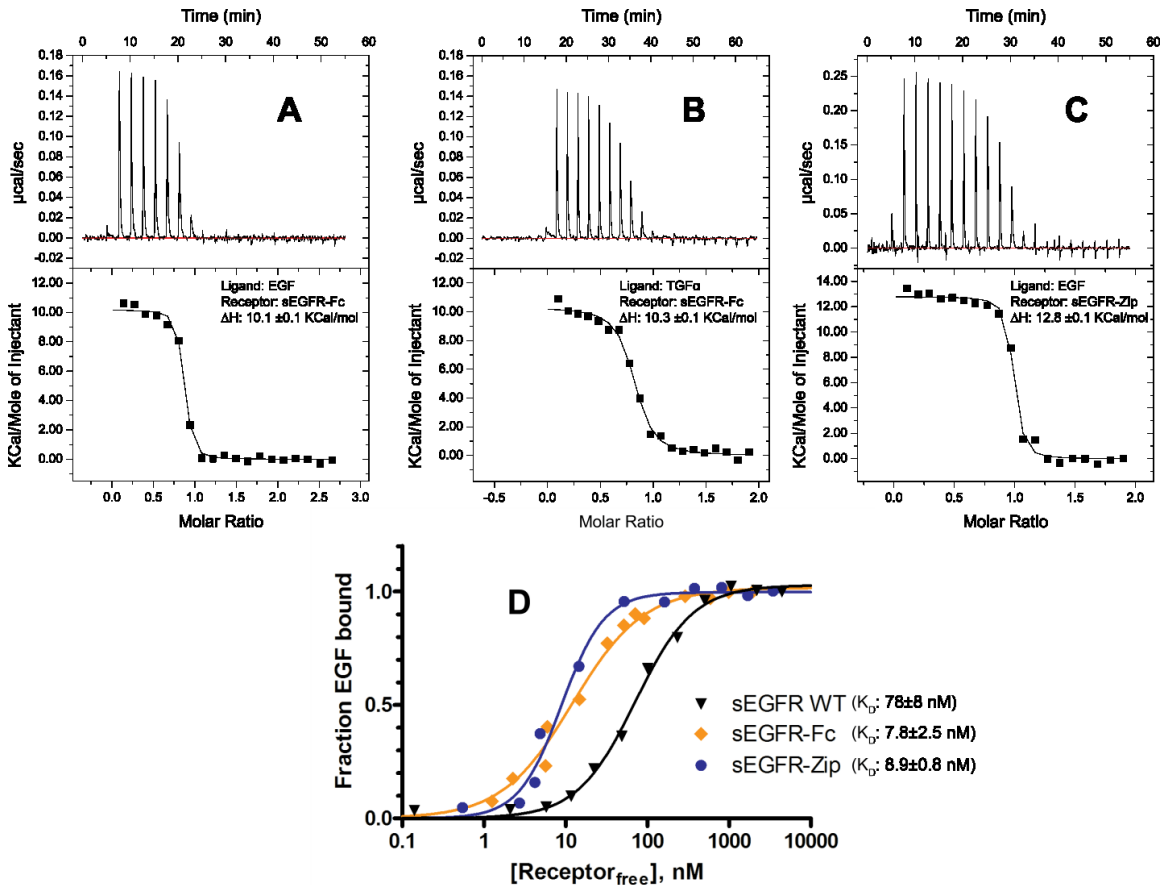
Figure 8: ITC Titrations: A: EGF titrated into sEGFR-Fc B: TGFα titrated into sEGFR-Fc C: EGF

titrated into sEGFR-Zipper D: Fluorescence anisotropy experiments, in which receptor variants

are effectively titrated into a solution of Alexa-488-labeled EGF

Table 2

EGFR variant	Ligand	$K_D^{\text{Ligand-induced dimerization}}$, μM	$K_D^{\text{Ligand-binding}}$, nM	$\Delta H^{\text{Ligand-binding}}$, kcal/mol
Fc fusion	EGF	Constitutive dimer	7.8 ±2.5	+10.1 ±0.1
	TGFα	Constitutive dimer	not determined	+10.3 ±0.2
Zipper fusion	EGF	Constitutive dimer	8.9 ±0.8	+12.8 ±0.2

To better understand the origin of increased ligand affinity for pre-dimerized receptor, we again exploited ITC, titrating EGF into sEGFR-Fc (Fig. 8A) and sEGFR-Zip (Fig. 8C), or TGFα into sEGFR-Fc (Fig. 8B). All titrations into pre-dimerized receptor showed an increased positive (unfavorable) enthalpy of binding relative to wild-type EGFR (Table 2). Given the increased affinity of ligand for pre-dimerized EGFR, the $\Delta S^{\text{ligand-binding}}$ must be significantly increased (more favorable) for pre-dimerized receptor. This sort of entropic effect is easy to imagine, given that the pre-formed dimer will likely be more highly-ordered, exhibiting fewer degrees compared to the wild-type, monomeric receptor. Whereas locking the wild-type (unconstrained, monomeric) receptor into an ordered dimer upon ligand-binding is predicted to be strongly-disfavored entropically, the entropic consequences of doing this for pre-dimerized sEGFR should be far less severe, since much of the entropic penalty will have been paid in forcing the dimer. Thus, we conclude that pre-dimerized EGFR binds ligand more tightly by minimizing the entropic penalty of receptor dimerization.

Pre-dimerized EGFR exhibits two distinct binding sites in a temperature-dependent manner

Upon closer inspection of titrations of EGF into sEGFR-Fc performed at 25°C we realized that they were ambiguous in the number of distinct binding sites that they suggested were present on the dimer. In order to resolve this issue, we performed a series of EGF titrations into sEGFR-Fc over a temperature range of 10°C to 30°C (Fig. 9). Although most titrations employed 10 μM protein in the ITC cell, we also performed a 25°C titration of sEGFR-Fc at 25 μM in order to increase signal-to-noise and improve confidence in our fitting (the considerable amount of

protein required prohibited us from using this concentration at other temperatures). As a control, we performed titrations of EGF into sEGFR^{WT} from 10^{0}C to 25^{0}C.

Temperature
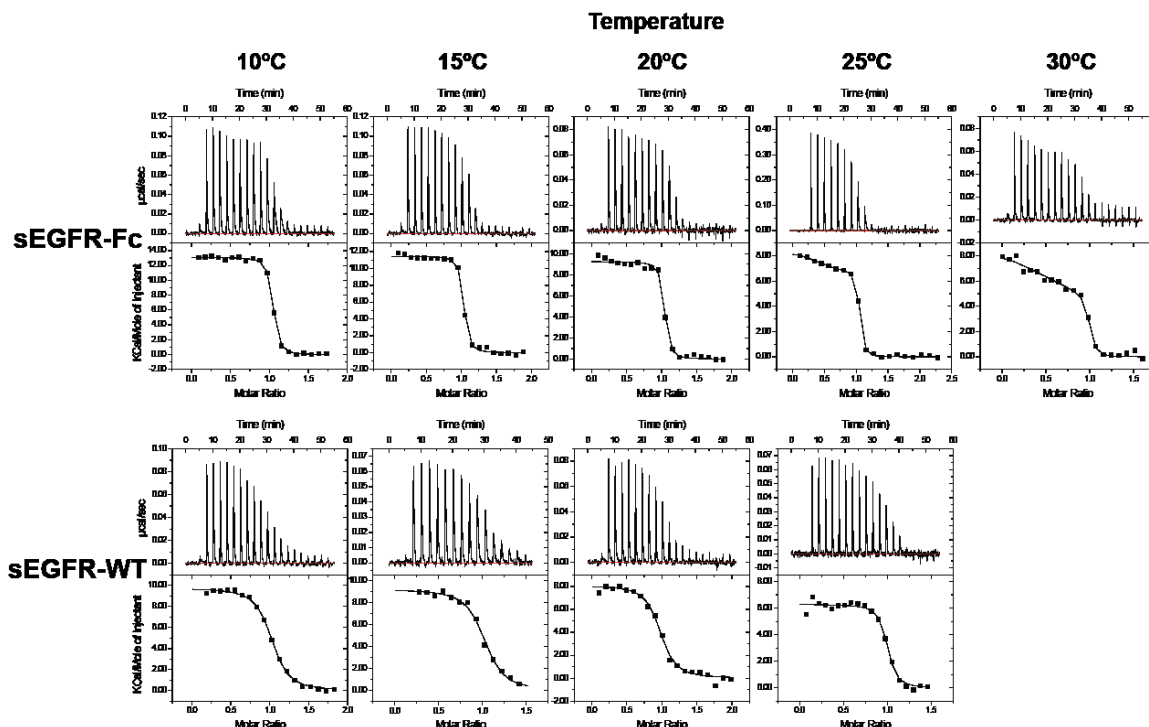
Figure 9: *ITC titrations of EGF into sEGFR-Fc and sEGFR^{WT} from 10-30°C. EGF titration into sEGFR^{WT} at 30°C was not performed. Unless otherwise noted, [receptor] was at 10µM in the ITC cell to begin the experiment, and data were fit to a standard single-site binding model. Note: for sEGFR-Fc at 25°C, a 25µM [receptor] was used to improve signal-to-noise for more confident fitting; additionally, sEGFR-Fc titrations at 25°C and 30°C demanded a two-site binding model to obtain good fits to the data. In each case, fitting to a two-sites binding model suggested equal stoichiometry for both sites, but the Kd and ΔH for each site can not be fit with precision. The equivalent stoichiometry for each site suggests that these sites represent two independent ligand-binding sites on an sEGFR-Fc dimer, and not a receptor dimerization event.*

The 25 µM sEGFR-Fc titration at 25˚C (Fig. 9), with improved signal-to-noise, argues that two distinct events are represented in the curve – justifying fitting of the data to two distinct binding

sites. This conclusion is further strongly supported by the sEGFR-Fc titration at 30^{0}C. The appearance of two distinct binding sites, with different $\Delta H^{ligand-binding}$ values but equivalent stoichiometries (that sum up to a 1:1 EGF:sEGFR-Fc ratio) argues that, at temperatures above 20^{0}C, the two binding sites in an sEGFR-Fc dimer become inequivalent, suggesting asymmetry. Unfortunately, it is not possible to fit the distinct $\Delta H^{ligand-binding}$ and K_D values for the two with any precision, because there are too many variables for the number of possible observables. In contrast to sEGFR-Fc, sEGFR^{WT} can be fit confidently with a single-site binding model throughout the temperature range tested (10^{0}C to 25^{0}C). This argues that enforced dimerization at the C-terminus of the EGFR ECR (as is likely to occur in the full-length receptor, due to the intrinsic dimerization affinities of the tyrosine kinase and transmembrane domains) can be sensed by the ligand-binding sites on the receptor dimer.

To examine whether the complex ligand binding we observed for sEGFR-Fc depends on allosteric communication of the two binding sites through the receptor domain II dimerization interface, we performed SPR and ITC binding studies with sEGFR-Fc bearing the Y251A/R285S double mutation that abrogates dimerization via domain II (abbreviated 'OgFc' in Figure 10C). As shown in Fig. 10A and B, this mutation did not significantly affect the thermodynamics of EGF binding in ITC. However, SPR binding data clearly show that the affinity of TGFα for sEGFR-Fc is affected by alterations inn the domain II dimerization interface; sEGFR-Fc$^{Y251A/R285S}$ binds TGFα with an affinity that is 6-fold lower compared to sEGFR-Fc (Fig. 10C). This result argues that the ligand-binding sites within sEGFR-Fc are sensitive to domain II-mediated interactions between protomers. It is interesting that this effect is ligand-dependent. EGF binds sEGFR-Fc in SPR studies with equivalent affinity regardless of the dimerization interface (Fig. 10C). We conclude from these studies that pre-dimerized sEGFR – but not sEGFR^{WT} – exhibits complex ligand-binding (which is consistent with weak negative cooperativity) at temperatures approaching physiological body temperature. This observation may underlie the difference in binding behavior seen for human EGFR when studies of isolated, monomeric extracellular regions are compared with those focused on cell-surface EGFR.

A.

10 µM sEGFR-Fc-WT
ΔH=10.2±0.2 kcal/mol

B.

10 µM sEGFR-Fc-Dim*
ΔH=9.5±0.2 kcal/mol
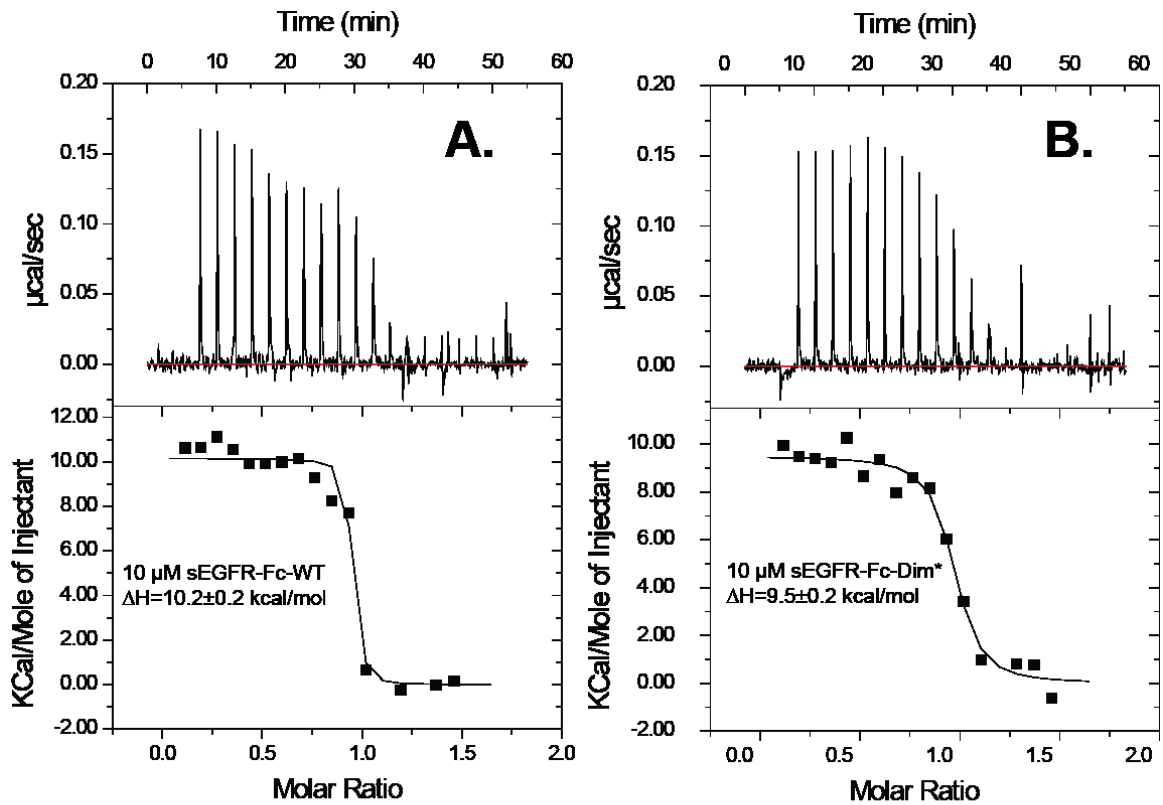
C.

SPR: sEGFR-Fc^{WT} and sEGFR-Fc^{Dim*} ligand-binding

WTFc EGF K_D~5nM
WTFc TGF K_D~4nM
OgFc EGF K_D~4nM
OgFc TGF K_D~23nM

38
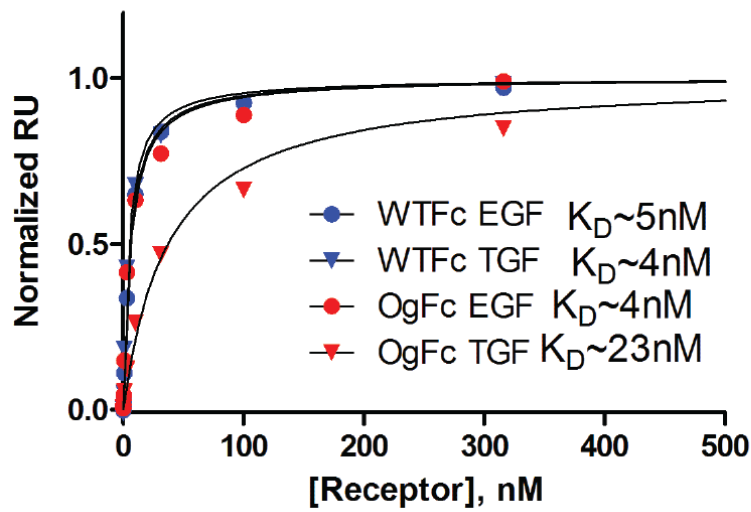
Figure 10: *A-B: ITC titrations of EGF into 10µM sEGFR-Fc (A.) and 10µM sEGFR-Fc$^{Y251A/R285S}$ (B.), which bears a compromised domain II dimerization interface. For simplicity, titrations were fit to a single-site binding model. C: SPR binding curves for sEGFR-Fc ('WTFc') and sEGFR-Fc$^{Y251A/R285S}$ ('OgFc'), flowed over a surface bearing EGF and TGFα on two different flow cells. Curves were fit to a single-site specific binding model in the Prism software, and fitted K_D's are displayed.*

Domain II:Domain IV intramolecular interactions stabilize inactive EGFR enthalpically

The minimal ligand-induced receptor dimerization model depicted in Figure 3 initially led to the hypothesis that the intramolecular tethering interactions between domains II and IV of the inactive monomeric EGF receptor serve a crucial auto-inhibitory function (Burgess, 2003; Ferguson et al, 2003). It was proposed that this auto-inhibition occurs through stabilization of the tethered form of the EGFR ECR, which is compatible with neither high-affinity ligand-binding, nor the domain II-mediated dimerization required for receptor activation. Previous studies showed that mutations compromising the tether do indeed increase the ligand-binding affinity of the receptor, yet these receptor mutants are not constitutively-active in cells(Walker et al, 2012), and SAXS studies have shown that isolated sEGFR bearing these mutations maintain a compact, monomeric conformation incompatible with domain II-mediated dimerization(Dawson et al, 2007). Although this proposed model for EGFR autoinhibition anticipated that activating oncogenic mutations might be found that disrupt autoinhibitory domain II/IV tether interactions, none have ever been found. Instead, cancer-causing ECR mutations that activate EGFR in vivo tend to cluster in the domain I/domain II interface (Lee et al, 2006). These findings prompted us to test the view that the tether interaction stabilizes the inactive, compact form of the receptor. To do so, we examined the thermodynamics of ligand-binding for a series of receptor ECR variants in which

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the tether is compromised to varying degrees and which have different receptor dimerization affinities.

Based on known structures, we would predict that mutations which disrupt the tether interaction should make $\Delta H^{ligand\text{-}binding}$ more favorable by removing electrostatic and van der Waals interactions that enthalpically stabilize the unbound (tethered) state of the receptor (without contributing significantly to the dimerization). The EGFR truncation consisting of mature receptor residues 1-501 (sEGFR[501]) has long been known to bind EGF with high-affinity (Elleman et al, 2001) – indeed, when crystal structures first became available, it was clear that the complete abrogation of the tether interaction in sEGFR[501] is likely responsible for this effect. Because domain II remains intact, sEGFR[501] still dimerizes upon ligand-binding in the same manner as the wild-type receptor. We find that sEGFR[501] binds EGF with a $\Delta H^{ligand\text{-}binding}$ that is nearly 4 kcal/mol lower (more favorable) than wild-type receptor (Fig. 11A; Table 3). This indicates a more-favorable interaction, and is in good agreement with qualitative predictions from the crystal structure. We sought to further test the role of the tether with a more modest mutation, in which domain IV is still present, to rule out thermodynamic effects arising from domain IV, which is known to play a role in receptor homodimerization(Kumagai et al, 2001; Lu et al, 2010). The loop encompassing EGFR residues 575-584 constitutes a majority of the tether interaction surface on domain IV. Deletion of this loop is known to increase ligand-binding affinity without a dramatic effect on ligand-induced dimerization(Dawson, 2005). As with sEGFR[501], we find that EGFR[Δ575-584] exhibits an enthalpy of ligand-binding that is reduced (more favorable) relative to wild-type, by 3.4 kcal/mol (Fig. 11B).

Table 3

EGFR variant	Ligand	$K_D^{Ligand\text{-}induced\ dimerization}$, μM	$\Delta H^{Ligand\text{-}binding}$, kcal/mol
1-501	EGF	~3	+2.2 ±0.2
Δ575-584	EGF	~2	+2.7 ±0.1
246-253*	EGF	>200	+3.3 ±0.1
246-253*/Δ575-584/D563A/H566A/K585A	EGF	>200	+2.5 ±0.1
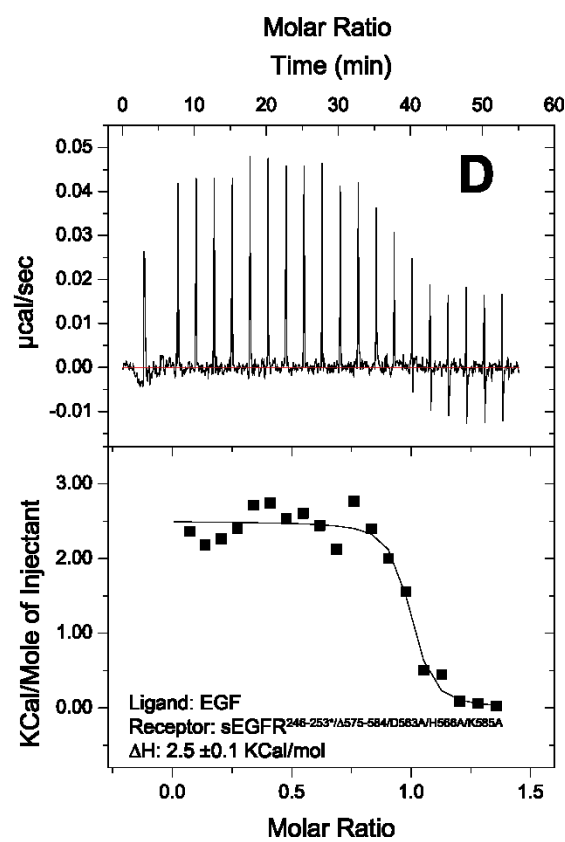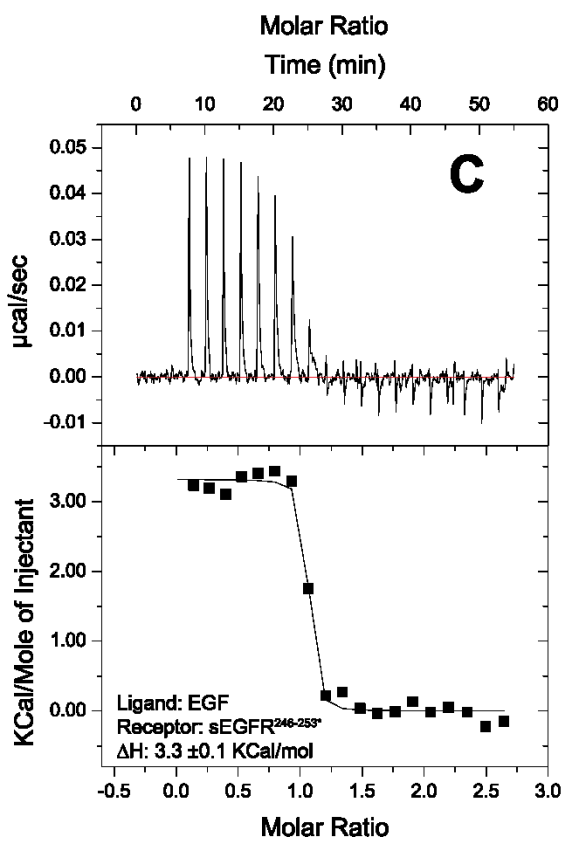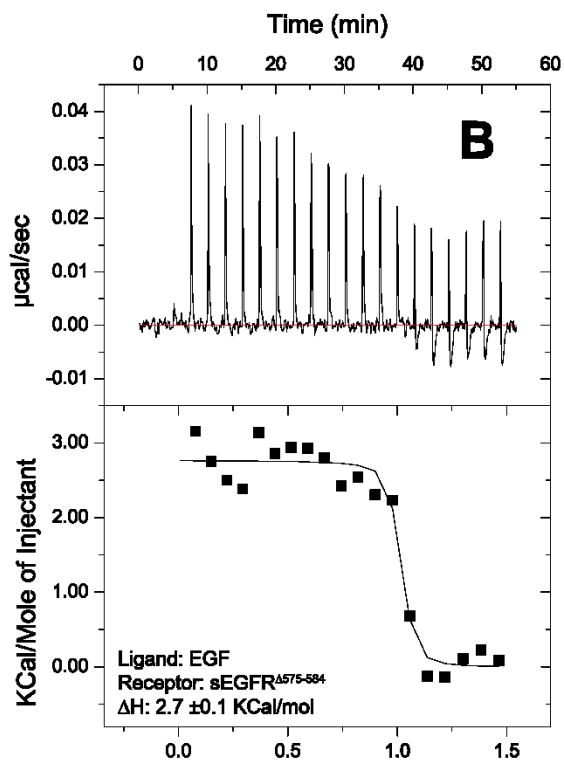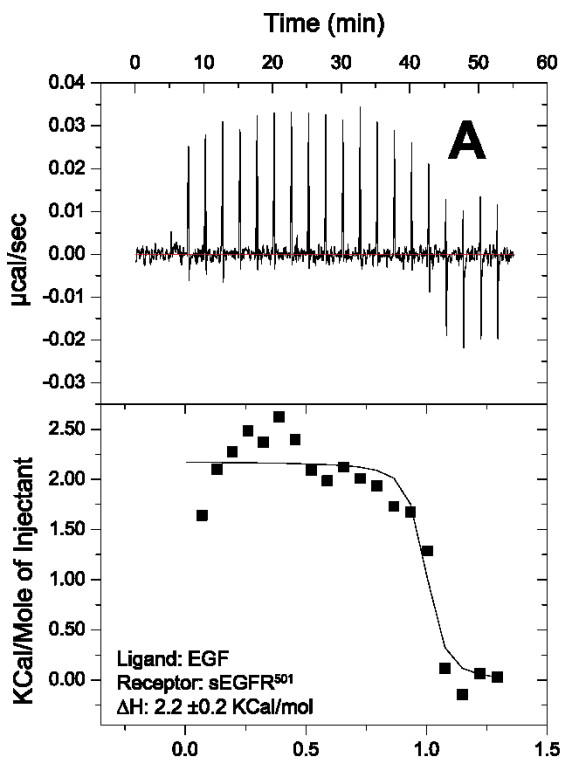
Figure 11: *ITC titrations of EGF into receptor variants A: sEGFR[501] B: sEGFR[Δ575-584] C: sEGFR[246-253*] D: sEGFR[246-253*/D563A/H566A/Δ575-584/K585A]*

The domain II surface that contributes to the inhibitory tether also constitutes the primary site of homodimeric interaction in ligand-bound receptor dimers. A set of six point mutations in this region (Y246D/N247A/T249D/Y251E/Q252A/M253D) has previously been described, which disrupts both the tether interaction and ligand-induced dimerization(Garrett, 2002). Like the truncation (sEGFR[501]) and domain IV (sEGFR[Δ575-584]) mutations just described, a receptor protein incorporating these six mutations (sEGFR[246-253*]) binds EGF with a $\Delta H^{ligand\text{-}binding}$ much lower (more favorable) than that of wild-type sEGFR, although the magnitude of the effect is slightly less, at 2.8 kcal/mol (Fig. 11C). Combining these six point mutations in domain II with the domain IV tether-loop deletion and three additional domain IV tether-disrupting point mutations (sEGFR[246-253*/D563A/H566A/Δ575-584/K585A]) yields a receptor mutant that displays a similar decrease in $\Delta H^{ligand\text{-}binding}$ as other tether-disrupting mutants, at 3.6 kcal/mol (Fig. 11D). The striking similarity in thermodynamic and affinity effects of all four mutants described here emphasizes that our structural view of tether auto-inhibition is accurate, that domain IV contributes <1 kcal/mol to ligand-binding and dimerization, and that $\Delta H^{dimerization}$ is insignificant compared to $\Delta H^{ligand\text{-}binding}$.

Transforming mutations in the EGFR ECR do not drive receptor dimerization

The tether mutants described above were originally predicted to activate EGFR when the structures depicted in Figure 3 were first determined. They fail to do so. In contrast, several unexpected transforming mutations in the EGFR ECR were more recently discovered in glioblastoma tissue samples(Brennan et al, 2013; Lee et al, 2006). Although numerous transforming mutations have been reported in the intracellular region of EGFR, most notably in lung cancer, these glioblastoma mutations are the first oncogenic driver mutations reported in the extracellular region. EGFR bearing these mutations has been shown to transform NIH-3T3 cells and to increase proliferation in Ba/F3 cells (Lee et al, 2006). These glioblastoma-causing

mutations cluster strikingly in the regions of EGFR domain I and domain II that form an intra-molecular interaction surface in the tethered, inactive receptor (Fig. 13B). In the ligand-bound, active form of the receptor, this intra-molecular interaction is subtly interrupted (lower part of Fig. 13B). To investigate the consequences of these mutations and understand how they lead to EGFR activation, we have examined the linkage of ligand-binding and dimerization for the most frequently-detected extracellular EGFR driver mutations in glioblastoma: R84K, A265D, and A265V.

Because EGFR activation requires dimerization of the intracellular kinase domains, we hypothesized that activating extracellular mutations act simply by causing receptor dimerization in the absence of ligand. Surprisingly, however, equilibrium analytical ultracentrifugation analysis shows clearly that sEGFR variants harboring any of the three tested mutants remain monomeric in solution (Fig. 12A). Addition of TGFα results in dimer formation, with dimerization affinities equivalent to that of WT EGFR ECR. It is clear that these mutations do not drive dimerization of unligated sEGFR to an appreciable extent (Fig. 12A; Table 4).
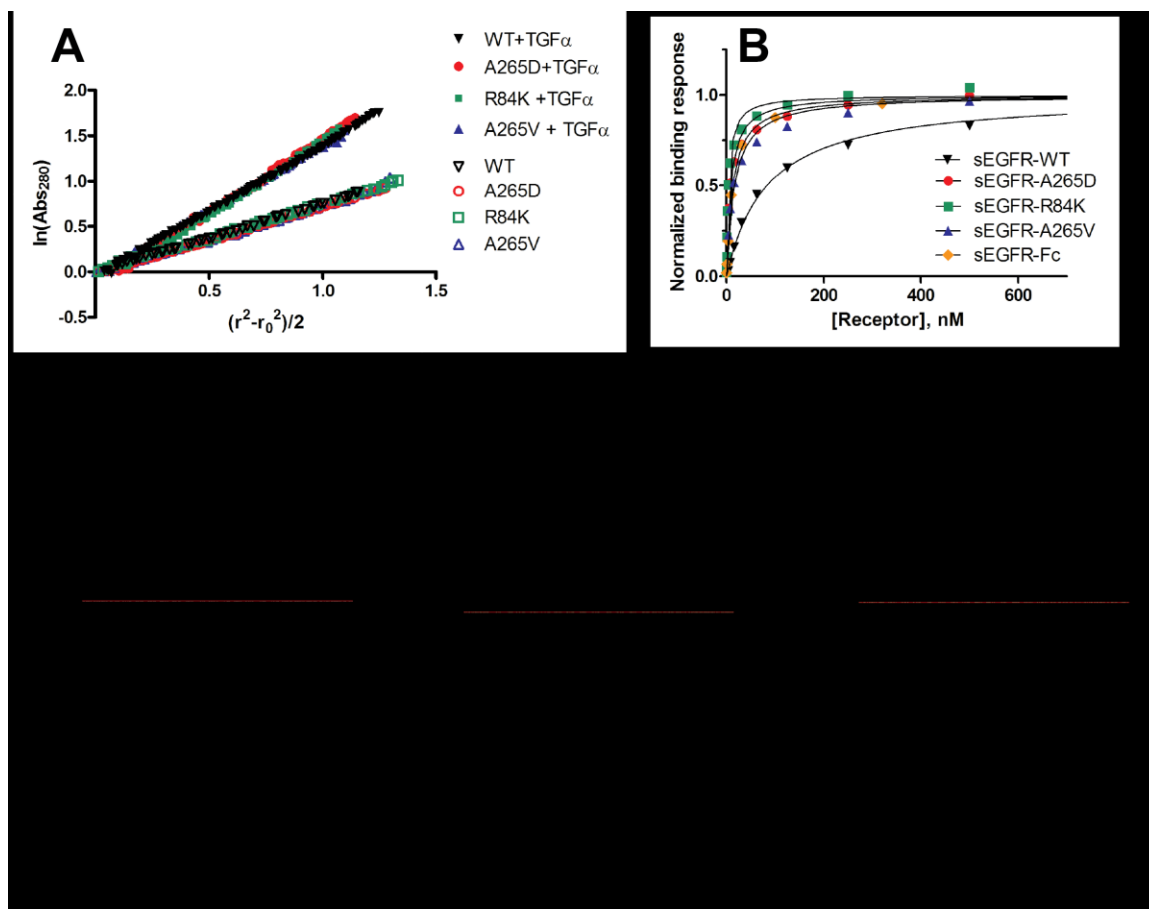
Figure 12: *A: Sedimentation equilibrium AUC data at 9000 RPM for glioblastoma mutants plus and minus TGFα B: SPR binding curves for glioblastoma mutants over an EGF surface; sEGFR-Fc has been included as a control C-E: ITC titrations of EGF into: C: A265D D: R84K E: A265V*

Table 4

EGFR variant	Ligand	$K_D^{\text{Ligand-induced dimerization}}$, μM	$K_D^{\text{Ligand-binding}}$, nM	$\Delta H^{\text{Ligand-binding}}$, kcal/mol
R84K	EGF	Not determined	4.3 ±0.9	+3.7 ±0.1
	TGFα	1.5 ±0.6	8.6 ±0.8	Not determined
A265V	EGF	Not determined	16 ±4.7	+4.1 ±0.1
	TGFα	1.0 ±0.2	89 ±6.0	Not determined
A265D	EGF	Not determined	8.6 ±3.0	+8.2 ±0.1
	TGFα	4.8 ±0.6	55 ±4.0	Not determined

Whether these mutations activate EGFR by enhancing constitutive dimerization or not, we expect – given their location in the domain I/II interface – that they might alter the thermodynamics of ligand binding. Indeed, mutations in this region of the *Drosophila* EGFR enhance its affinity for its ligands (Alvarado, 2009). A mutated EGFR that has elevated ligand-binding affinity could be more 'ligand-sensitive', becoming activated by low 'background' levels of ligand that do not significantly activate WT EGFR. Indeed, given that residues R84 and A265 in EGFR contribute to an intra-molecular interaction that is specific to the tethered form of the receptor, one would predict that mutating these residues might increase ligand-binding affinity (Figure 13B). To test this hypothesis, we measured binding of sEGFR[R84K], sEGFR[A265D], and sEGFR[A265V] to EGF and TGFα using SPR. All of these mutant forms bind to ligand with an elevated affinity (Fig 12B and Table 4). Based on analysis of ligand-binding and ligand-induced dimerization, sEGFR[R84K], sEGFR[A265D], and sEGFR[A265V] are strikingly similar to the tether-disrupting mutants described above; however, they transform cells and cause cancer, whereas the tether-disrupting mutations have not been observed to do so.

To extend this comparison, we performed ITC titrations to determine the energetic basis of ligand-binding to sEGFR[R84K], sEGFR[A265D], and sEGFR[A265V]. Based on the structural model presented in Figure 3 and in analogy to tether-disrupting mutations, we would predict that each of these glioblastoma-derived point mutations increases ligand-binding affinity by enthalpically destabilizing the tethered state, resulting in a decreased (more favorable) $\Delta H^{ligand-binding}$ for ligand binding to these mutants. While this prediction holds true for sEGFR[R84K] and sEGFR[A265V], where $\Delta H^{ligand-binding}$ is decreased (more favorable) by 2.0-2.4 kcal/mol, sEGFR[A265D] actually has an increased (*less* favorable) $\Delta H^{ligand-binding}$ compared to sEGFR[WT], by 2.1 kcal/mol (Fig. 12C-E, Table 1). These results clearly point to the domain I/domain II interface as an important site of autoinhibitory interactions in human EGFR – and one that is thermodynamically just as significant as the much discussed domain II/domain IV tether. However, the specific effects on $\Delta H^{ligand-binding}$ argue that the effect of mutating the domain I-domain II interface is complex, and cannot readily

be explained by the simple model presented in Figure 3. More exploration of this interface is required, in parallel with analysis of more patient-derived mutations.

The complex functional consequences of these mutations is consistent with cellular data showing that these mutations do increase basal EGFR signaling, while still retaining some ligand-dependence. It is difficult to fully exclude effects on receptor dimerization, since we would not detect unligated ECR dimerization with a K_D above ~10-20 μM in these studies. A small increase in self-association affinity of the mutated ECR might be sufficient, combined with TKD and TM domain dimerization, to shift the balance towards significant constitutive dimerization of the intact EGFR in cells. Synthesis of these data suggests that these mutations may, for example, be capable of tipping the equilibrium within ligand-independent EGFR dimers toward a more active signaling state, without strongly shifting the overall monomer-dimer equilibrium of the receptor ECR.

Conclusions

A great deal of investigation to this point, spanning from in vitro biochemistry and structural biology to cellular studies, has bolstered the view that dimerization is a crucial determinant of signaling within the ErbB receptor family – and throughout the larger family of receptor tyrosine kinases (RTKs)(Lemmon & Schlessinger, 2010). EGFR-activating ligands drive receptor dimerizatio, aberrant receptor dimerization drives activation in many contexts, and preventing receptor dimerization with mutations or therapeutic agents plainly prevents receptor signaling. Accordingly, early reports of ligand-independent, inactive EGFR dimers within cells were met with uncertainty(Gadella & Jovin, 1995; Sako et al, 2000; Yu, 2002). Recent crystallographic data, however, have captured a distinct inactive EGFR dimer that gives credence to the notion of physiologically-relevant, ligand-independent EGFR dimers(Alvarado, 2009). This notion has clear precedent in the RTK family; insulin receptor forms a constitutive covalent dimer that remains inactive until insulin is bound(McKern et al, 2006; Menting et al, 2013), with activation presumably ensuing through intra-dimer conformational changes that have yet to be fully understood.

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The outdated view that ligand-induced dimerization is the *sole* determinant of receptor activity (as depicted in Figure 3) has been invoked to argue that the primary function of EGFR ligands must be to induce receptor dimerization. An important corollary of this simple argument is that ligand must bind more tightly to dimerizing receptor than to monomeric receptor. Our binding data clearly show that the EGFR ligands EGF and TGFα bind monomeric and dimerizing receptor with equivalent affinities, and this data is supported by recently published cellular data (MacDonald, 2008). The enthalpy of ligand-binding, however, is significantly different for monomeric sEGFR compared to the dimerization-capable sEGFR for both EGF and TGFα. The enthalpy data argue that EGF and TGFα bind to sEGFR to form an initial complex that is qualitatively distinct from one-half of a ligand-induced receptor dimer. The weak energetic coupling of ligand-binding and dimerization, conserved for EGF and TGFα, further argues that functional constraints other than driving EGFR homodimerization have driven ligand evolution. These data show that the qualitatively distinct and poorly understood conformation of a 1:1 ligand:EGFR complex (see Figure 13) is relevant to receptor function.

Figure 13A

INACTIVE
EGFR monomers

ACTIVE

INACTIVE
Pre-formed EGFR dimer
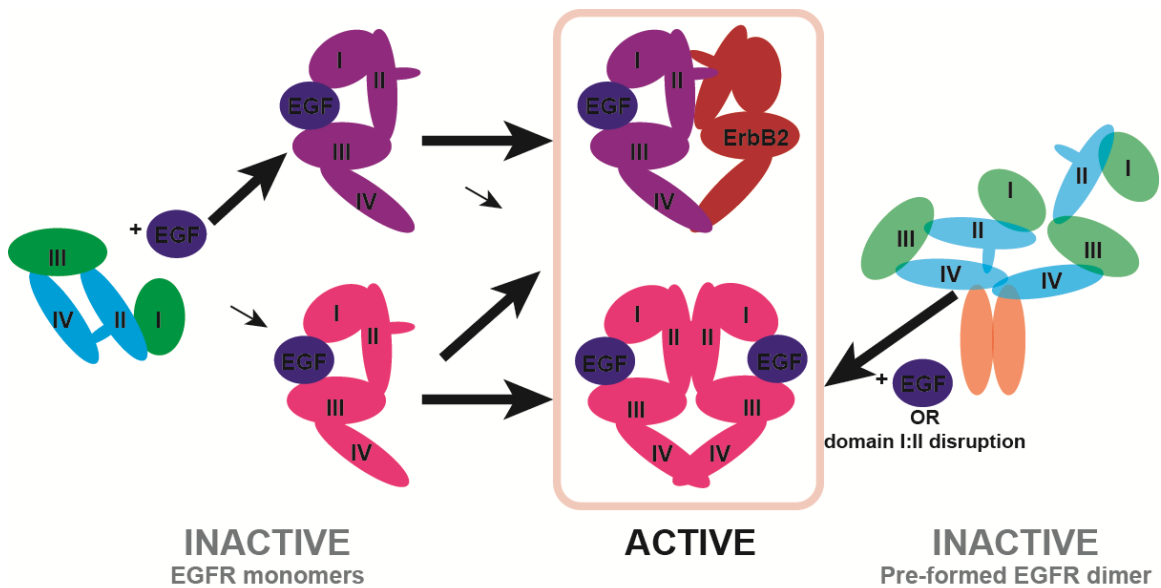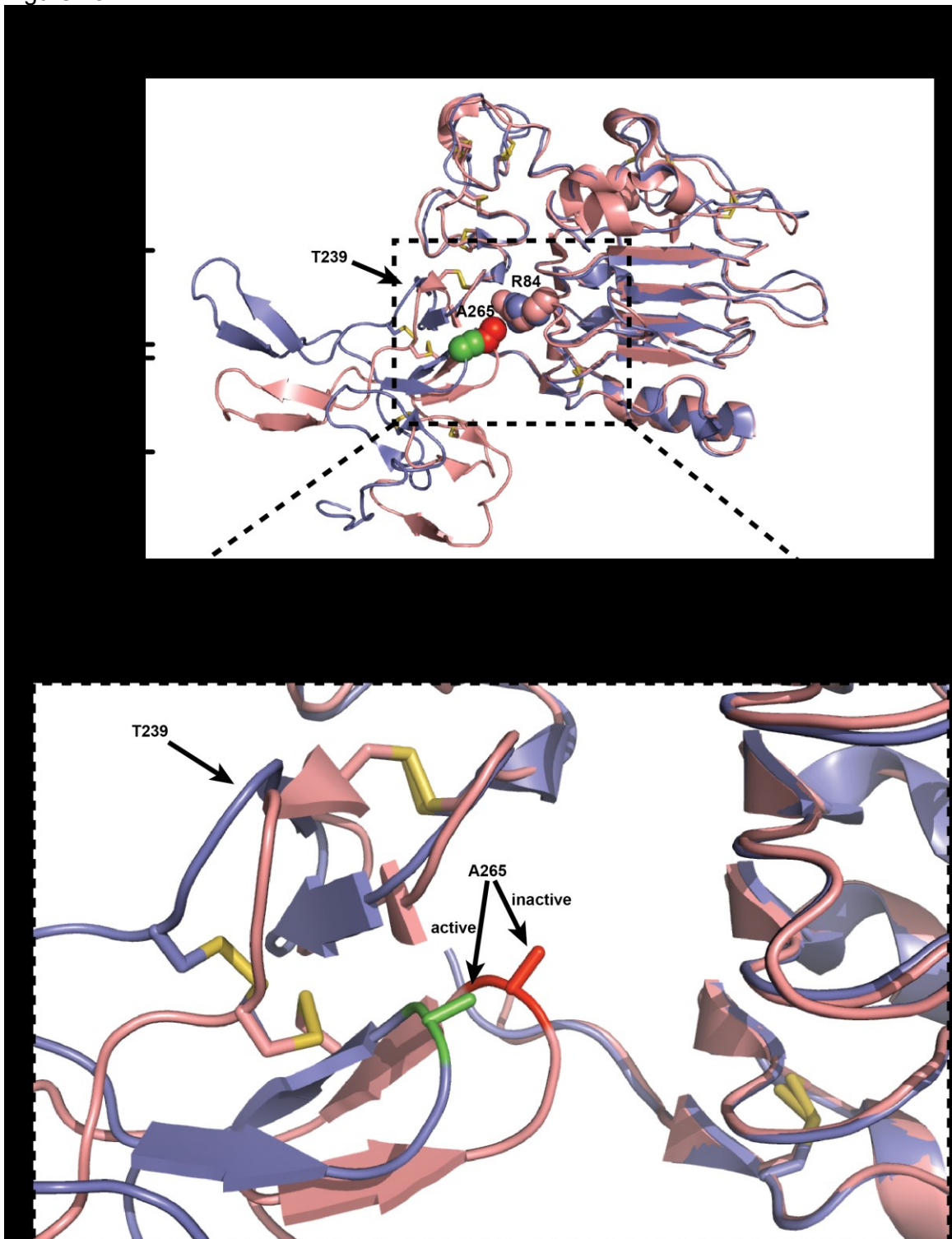
Figure 13B

Figure 13: *A: An expanded schematic model for ligand activation of EGF receptor, based on data presented here. When ligand binds to monomeric EGFR, it preferentially populates a receptor state (in purple) that is not configured for optimal receptor homodimerization. The true conformation of this state is not known at atomic resolution. This EGF:EGFR state favors binding to ErbB2 to yield an EGF:EGFR:ErbB2 heterodimeric signaling complex. Alternatively, EGF can bind (with a lower affinity) to a receptor state (in pink) that is identical to the EGFR state within a homodimer. Pre-formed, inactive EGFR dimers (at right) are activated by a related but unique pathway; in the absence of ligand, the extracellular regions of the receptor do not form an ordered dimer. Ligand-binding induces formation of the receptor domain II-mediated extracellular dimer. B: Comparison of active (blue; PDB 1IVO) and inactive (pink; 1YY9) crystal structures points to an allosteric regulatory role for the domain I:domain II interface in ligand-binding and dimerization. A similar alignment with all three known structures of ligand-activated ErbB receptors and all four known structures of inactive EGFR and ErbB4 (not shown) illustrates that this pattern is strictly conserved. These alignments show exquisite structural conservation of the A265 sidechain, which is buried in a hydrophobic interface between domain I and domain II. Mutation of this sidechain to a bulky residue is predicted to structurally disrupt this interface; to accommodate extra methyl- or carboxyl- groups, the protein backbone in the A265 region must translate away from domain I, mimicking the structural effect of ligand-binding in this region. Although the R84 sidechain is less-conserved structurally, it can form hydrogen bonds with domain I and domain II, providing a structural bridge between these domains which may be disrupted upon mutation.*

What is the nature and the functional relevance of a 1:1 ligand:EGFR complex? Shaw and colleagues have run microsecond molecular dynamics simulations that suggest such a complex forms a tethered-like structure in which a (previously unobserved) kink occurs near the N-terminus of domain IV(Arkhipov et al, 2013). Accordingly, they have proposed a model (consistent with binding data presented here) where the primary function of ligand is not to drive receptor dimerization through domain II, but rather to eliminate steric autoinhibition of a TM- and

kinase-domain-driven dimer. Such a model, however, cannot account for the ability of a solitary extracellular point-mutation (Y246D) in the domain II dimerization interface to completely abrogate ligand-induced receptor signaling(Walker et al, 2012) – which argues that domain II-mediated dimerization is crucial for receptor signaling activity. Moreover, one complication in interpreting the Shaw laboratory simulations is that they predict the stability of complexes (such as unligated sEGFR dimers) that have never been observed experimentally – indicating that key elements have been misestimated.

A more satisfactory explanation for the functional importance of a 1:1 ligand:EGFR complex recalls the primary importance of heterodimeric EGFR signaling complexes. Indeed, it has long been thought that EGFR ligands will preferentially signal through EGFR:ErbB2 heterodimers when applied to cells expressing the full complement of ErbB receptors. These observations sparked the notion that this preference may arise from a simple biophysical preference of ligand for the heterodimer; experiments utilizing isolated receptor ECRs, however, argued against this simple hypothesis (Ferguson, 2000). Importantly, more recent measurements have been made in the context of full-length receptors expressed in cells. Here, EGF binds more tightly to EGFR within an EGFR:ErbB2 heterodimer, relative to an EGFR homodimer(Li et al, 2012) – presumably reflecting contributions of the TM and intracellular domains (as well as possible membrane contributions) to receptor heterodimerization. We propose that the 1:1 ligand:EGFR complex in solution adopts a conformation (Figure 13A, purple) mimicking that within a ligand:EGFR:ErbB2 complex in cells. This could explain the need for EGF and TGFα to maintain a high affinity for monomeric EGFR. Binding data presented here, combined with exciting new data from cellular studies, thus calls for a more detailed investigation of ligand binding to monomeric and heterodimeric EGFR.

We initially hypothesized that ligand-independent EGFR dimers mimic those observed crystallographically for the *D. melanogaster* EGF receptor (dEGFR). By fusing dimerizing domains to the EGFR ECR, we created ligand-independent EGFR dimers in solution. These dimers, surprisingly, still require bound ligand to form a well-ordered, domain II-mediated dimer

interface. Preliminary ligand-binding data suggest that the EGFR ECR within sEGFR-Fc is

significantly tethered (data not shown). Thus, these forced human sEGFR dimers do not seem to

mimic dEGFR. It is clear from the high affinity of these dimeric fusion proteins for ligand, and the

EM images of the dimeric complexes that they form, that they are perfectly compatible with the

crystallographically-observed active dimer structure depicted in Fig. 3C, and that our fusion

approach has not compromised the receptor.   These observations, in combination with the high

local concentration of EGFR ECR within sEGFR-Fc (at least ~1 mM, and potentially much higher

depending on orientation effects) provide the strongest argument to date that ligand binding to the

EGFR ECR actively remodels the domain II dimerization interface into one capable of dimerizing,

rather than simply capturing a transient extended EGFR conformation. The long-standing

observation that sEGFR[501] (in which domain II should be free to dimerize) fails to dimerize in the

absence of ligand, further bolsters this view. Active allosteric remodeling of receptor by ligand,

specifically within the dimerization interface, is an important prerequisite for models of ligand-

specific signaling in which unique ligands induce subtly different receptor states or select for

specific heterodimer signaling complexes(Wilson et al, 2009). Accordingly, our data (contrary to

the minimal model in Fig. 3) suggest that such models are indeed feasible – and that there is

much more to ErbB activation that just ligand-induced dimerization. Available crystal structures

do show some differences in domain II conformation that depend upon ligand. For example,

ligand-binding induces or stabilizes a curvature along the long axis of domain II that facilitates

symmetric homodimer contacts (Fig. 13B). Unfortunately, the small magnitude of these

differences, the relatively low resolution of the available structures, and the lack of multiple

different ligand-complex structures for a given ErbB receptor protein has prevented more

sophisticated structural analysis of ligand-induced allostery in domain II so far. For example, a

structure of a monomeric ligand-bound EGFR would provide insight into whether structural

changes in domain II are primarily driven by ligand-binding or by homodimerization. Intriguingly,

it has been reported that an ErbB ligand bearing a specific point-mutation can function as a

receptor antagonist(Wilson et al, 2012b). Furthermore, recent data from conformation-specific

fluorescent reporters have suggested that different EGFR ligands (EGF vs TGFα) induce unique

structures in the intracellular regions of the receptor(Scheck et al, 2012). Additionally, ligand-

specific phosphorylation-site usage and cellular output has been reported(Wilson et al, 2012a).

Ligand-specific domain II-mediated receptor dimers would provide the most restrained

explanation for these observations, and further suggest that an approach of modifying ErbB

ligands – selecting for either inhibitory or (very specific) agonist activities (for example, stimulating

EGFR:ErbB2 heterodimers but not EGFR homodimers, or vice versa) – may yield useful reagents

for modulating ErbB signaling experimentally and therapeutically.

Another prediction of the minimal structural model in Fig. 3 is that activating mutations in

the EGFR extracellular region must act by simply inducing receptor dimerization. We report here

that the most common extracellular EGFR transforming mutations (R84K, A265V, and A265D) fail

to drive sEGFR dimerization in the absence of ligand. On the other hand, these mutations clearly

enhance ligand-binding. Given that these mutations increase proliferation in Ba/F3 cells, in which

ErbB ligands are undetectable (and in which tether-disrupting EGFR mutations, which increase

ligand-binding affinity to a similar extent, are not transforming), these mutants likely exhibit a

mechanism of activation beyond simply increasing ligand-binding affinity. Accordingly, it is

important to note that the EGFR domain I/domain II interface, compromised by these activating

glioblastoma mutations, is an obvious potential route for allosteric communication between the

ligand-binding sites and the dimerization interface (Fig. 13B).

In inactive crystal structures, R84 can form a highly geometrically-constrained hydrogen bonding

network that bridges R84 and E60 on domain I to the backbone of residue C227 in domain II (Fig.

13B). Upon ligand-binding and dimerization, the hydrogen bond between R84 and C227 appears

to be compromised, with the interatomic distance increasing by 1Å. Perhaps more strikingly, the

A265 sidechain is buried in a hydrophobic interface between domain I and domain II in the

inactive tethered conformation; ligand-binding and dimerization wedges domain II outward,

exposing the A265 side-chain to solvent. Furthermore, in the alignment in Figure 13B, the

inactive and active structures begin to diverge considerably around residue T239 and beyond;

though not studied here, a T239P mutation was also reported to cause EGFR activation and drive glioblastoma. We propose that the domain I/domain II interface represents an auto-inhibitory, allosteric interaction that maintains the domain II dimerization interface in an inactive, dimerization-incompetent conformation (Figure 13B). All of the oncogenic mutations described here could be rationalized by this model: the R84K mutation will eliminate a hydrogen-bond, weakening the linkage between domain I and domain II; mutating A265 to V or D will introduce a bulky side-chain that cannot be accommodated in the tightly-packed hydrophobic interface, effectively wedging the two domains apart much like the effect of ligand-binding; the T239P mutation will introduce atypical backbone geometric constraints in a hinge-like region that shifts upon activation. Accordingly, perturbing this interface generally may increase the basal activity of otherwise inactive pre-formed EGFR dimers (Figure 13) without necessarily driving receptor dimerization.

Taking a broad view of the data reported here, it becomes clear that EGFR ligands exhibit subtle and poorly understood allosteric regulation of EGFR function. As the primitive model depicted in Figure 3 has been largely debunked, the development and testing of more powerful structural models of ErbB signaling that can account for ligand-specific signaling outputs (either by ligand discrimination within a given homo- or heterodimeric complex, or by ligand selection of a specific signaling complex), complicated ligand-binding patterns, and inactive ligand-independent dimers is necessary. Our observations motivate further study (by showing that such allosteric regulation is not only feasible, but likely contributes to EGFR pathology) and offer a novel, refined model of ligand-dependent EGFR signaling.

CHAPTER 3: Characterizing signaling by diverse EGF receptor ligands

The data presented in Chapter 2 elucidate a complex mode of allosteric regulation well beyond the scope of the minimal structure-based model for ligand-induced dimerization. Where the minimal model fails to explain why seven different EGFR ligands are present in mammals, the allosteric regulation uncovered in this thesis hints that receptor complexes could potentially discriminate between the individual unique ligands. Unique ligands may induce unique receptor dimers upon binding – at the level of conformation or dynamics. Many observations in the literature are consistent with just such a mechanism.

Multiple studies have found ligand-dependent differences in receptor internalization rates(Roepstorff et al, 2009; Willmarth et al, 2009). This difference is particularly stark for the ligands Erg and Arg, which stimulate lower levels of receptor internalization than EGF. Since ligand-induced receptor internalization and degradation are largely mediated by receptor ubiquitination and c-Cbl binding at pY1045 on EGFR, these studies further examined these events. While both studies found differences in c-Cbl binding and ubiquitination for Arg-receptor complexes compared to EGF-receptor complexes, the two studies are not self-consistent; one reports increased ubiquitination of, but decreased c-Cbl binding to, Arg complexes, while the other reports decreased ubiquitination of Arg complexes. Both studies observe that the temporal stability of specific EGFR phosphotyrosines differs for Arg complexes compared to those induced by EGF. Importantly, neither of these studies has definitively pinpointed the origin of ligand-dependent ubiquitination and c-Cbl binding patterns. The simplest possible explanation for this difference would be unique, ligand-dependent receptor phosphorylation-site usage.

Further support for unique ligand-dependent receptor complexes comes from a structure-dependent imaging technique described by the Schepartz laboratory (Scheck et al, 2012). Briefly, a unique fluorescent reporter was employed that emits a fluorescent signal only when coordinated by four cysteine residues. Scheck et al. measured reporter fluorescence for a series of ligand-bound EGFR variants in cells, with cysteines inserted at various positions within the intracellular JM region. They report that EGF and HB-EGF receptor complexes showed high

fluorescence for one discrete set of EGFR cysteine variants, whereas TGFα complexes showed high fluorescence for a distinct set of EGFR cysteine variants. This observation argues that TGFα induces a unique intracellular receptor dimer structure compared to EGF and HB-EGF. Further work in cells has suggested that different ErbB ligands are differentially coupled to DNA synthesis, proliferation, and survival in cell culture (Hobbs, 2002; Wilson et al, 2012a).

While the studies summarized above are all consistent with ligand-dependent receptor complexes that possess unique signaling qualities encoded exclusively by the ligand-receptor complex, other possibilities must be considered. For example, the pH-dependence of ligand binding is known to affect receptor down-regulation, which may modulate steady-state receptor signaling at qualitative and quantitative levels (French et al, 1995). Accordingly, unique ligands may exert unique signaling effects by possessing unique pH-dependence profiles without inducing any unique receptor signaling state. Additionally, many of the studies cited above have employed cell lines expressing multiple ErbB receptors; ligand-dependent signals may be a result of unique receptor-binding specificities for the different ligands.

A primary challenge in understanding the capacity for EGFR to functionally discriminate between ligands has been the difficulty in obtaining reagents. EGF and TGFα are both readily expressed in *E. coli* and have been available from commercial suppliers for some time. Other ErbB ligands, however, do not express at high levels, and many years of work in the Lemmon laboratory and elsewhere has failed to produce large quantities of high-quality protein for biophysical studies. Remarkably, for biophysical studies of ErbB receptor signaling, generating the 50-100 amino acid ligand proteins has consistently been much more of a challenge than producing the 600-800 amino acid receptor extracellular regions. Although some of the ErbB ligands are nonetheless commercially available, cost is prohibitive for the sort of structural and biophysical experiments required to shed light on ligand-dependent allosteric effects. Moreover, the quality of expensive material that has been purchased by the Lemmon laboratory in the past has been found to be wanting – making this not a feasible route in any case.

To overcome these challenges, I have successfully employed a unique fusion protein approach (inspired by our experience with invertebrate ligands) to successfully express the ErbB ligands amphiregulin (Arg), epiregulin (Erg), and Epigen (Epg). The yields I have achieved are suitable for structural and biophysical analyses. I have observed that my recombinant Arg, Erg, and Epg are all capable of inducing EGFR phosphorylation in cell culture. Additionally, I have performed a series of ligand-binding studies with EGFR variants in analogy to studies described in Chapter 2. In results reminiscent of recent findings concerning ligand-binding to VEGF receptors (Brozzo et al, 2012; Leppanen et al, 2011; Leppanen et al, 2010), I find that the thermodynamics of ligand-receptor binding are highly ligand-dependent. This result stands in stark contrast to the results reported in Chapter 2, where the thermodynamics of receptor binding are strikingly similar for EGF and TGFα (with the exception of binding to the 'OgFc' variant, in Chapter 2, Fig. 10C). Finally, my results illustrate that Epg and Erg exhibit coupling to EGFR variants in a way that differs significantly from EGF and TGFα. My results thus suggest mechanisms by which unique ErbB ligands may elicit qualitatively unique receptor responses.

Recombinant production of bio-active EGFR ligands Epigen, Epiregulin, and Amphiregulin

Much effort in the Lemmon laboratory has been directed at the recombinant production of EGFR ligands in insect cell expression systems. Previous work indicated that the presence of the low-complexity leader sequence of Spitz (a ligand for *D. melanogaster* EGF receptor) was essential for high levels of recombinant protein production in Sf-9 cells. We reasoned that this leader sequence was required for the proper folding and secretion of EGF-like domains in insect cells. Accordingly, I made a series of plasmids encoding the EGF-like domains of human EGFR ligands following the Spitz leader sequence, with a protease-site for subsequent purification of the isolated EGF-like domains (Fig. 14A). This expression cassette was inserted into the pFastbac plasmid for generation of baculovirus for expression in Sf-9 cells, as well as the pMT vector for expression in S2 cells.
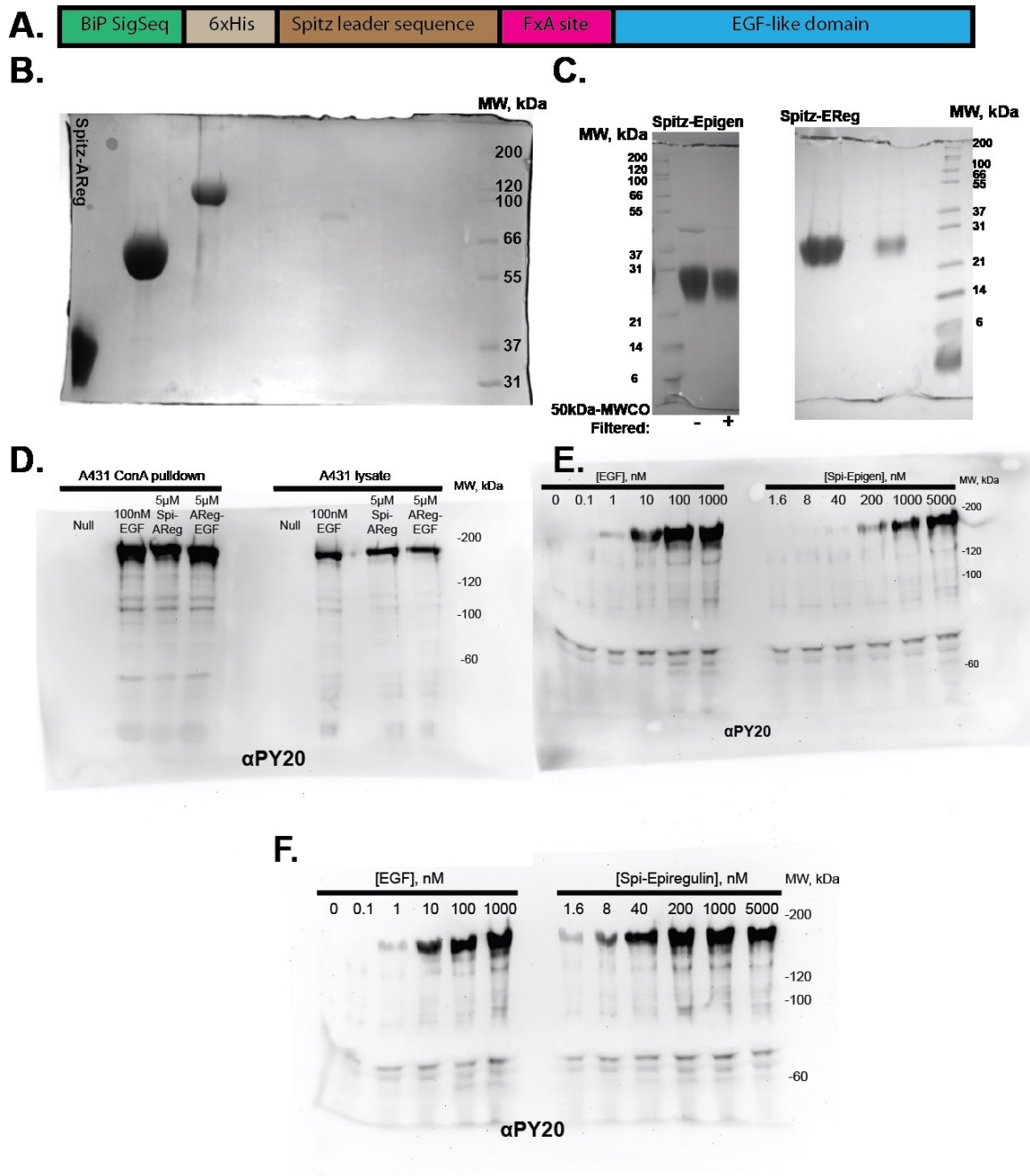
Figure 14: *Generation and validation of recombinant Arg, Erg, and Epg produced in insect cells.*

A: Architecture of the protein-coding constructs used for recombinant ligand production. B-C:

Coomassie-stained gels showing representative samples of purified Arg (B.) produced in Sf-9

cells, and Epg (C., left) and Erg (C., right) produced in Drosophila S2 cells. These gels show the

protein after Ni-NTA agarose, cation-exchange, and gel filtration purification steps. Erg and Epg

were further purified by sequential filtration through 50 kDa- and 30 kDa-molecular weight cutoff

protein concentrators (Amicon), which removed the majority of high molecular weight

contaminants (see Epg gel, +/- filtration step). Lanes D-F: Stimulation of EGFR-overexpressing

cell lines by recombinant ligands. 'Null' lanes were mock-stimulated with serum-free DMEM

media. D: A431 cells (which overexpress EGFR) were stimulated on ice for 10 minutes with

either 100nM EGF, 5µM Spitz-Arg, or 5µM Arg EGF-like domain alone ('Arg-EGF'), to ensure

saturation of receptor. Arg-EGF was created by FxA-mediated cleavage and subsequent NiNTA-

mediated removal of the Spitz leader sequence. Clarified lysates (right) and Concanavalin-A-

agarose pull-down samples (enriched for glycosylated proteins) were probed on a western blot

using the pY20 antibody, which broadly detects phosphotyrosine residues. E-F: MDA-MB-468

cells (which overexpress EGFR) were stimulated on ice for 10 minutes with increasing

concentrations of EGF, Epg (E.), and Erg (F.). Clarified lysates were probed on western blots

using the pY20 antibody.

I found that this Spitz-fusion approach allowed high-level expression of Arg in Sf-9 cells

(Fig 14B), and Erg and Epg in S2 cells (Fig 14C). For initial tests of signaling activity, I purified

the ligands by Ni-NTA agarose affinity, cation-exchange chromatography, and size-exclusion

chromatography. After these purification steps, the final yield for all three ligands is on the order

of 0.1-0.5 mg per liter of culture media – better than has been achieved in the laboratory in over

15 years of trying, and with much more homogeneous material. In order to test the activity of

these ligands, I incubated cell lines expressing high levels of EGFR on ice for 10 minutes with no

ligand ('Null' samples), or with purified Arg, Erg, or Epg, or with EGF as a positive control.

Ligands were tested at concentrations up to 5µM to ensure receptor saturation. Arg was tested

side-by-side with EGF on A431 cells, and both cell lysates and concanavalin-A pulldowns

(enriched for glycosylated proteins, including EGFR) showed a similar extent of EGFR activation

for Arg- and EGF-stimulated samples (Fig. 14D). Since Arg stimulated EGFR regardless of the presence of the Spitz leader sequence, subsequent studies with Arg, Erg, and Epg all left the Spitz leader sequence intact. Similarly, Erg and Epg were tested on MDA-MB-468 cells, and showed a similar extent of EGFR activation compared to EGF (Fig. 14E-F). These results illustrate that the ligands Arg, Erg, and Epg may be produced in high quantity and quality (as judged by signaling activity) in insect cell lines using the Spitz fusion approach.

Epigen, Epiregulin, and Amphiregulin bind to EGF receptor with unique thermodynamic profiles

EGF and TGFα exhibit exquisitely well-conserved thermodynamics in binding to constitutively-monomeric, constitutively-dimeric, and wild-type sEGFR. This level of conservation – in which the enthalpies, entropies, and free energies of ligand-binding are all conserved within 0.5 kcal/mol for all three receptor variants – seems to suggest that EGF and TGFα impart a well-conserved and functionally important allosteric regulation on EGFR. In contrast, the dependence of TGFα affinity upon the domain II dimerization interface of sEGFR-Fc hints that ligand-specific effects may be relevant for EGFR, since EGF affinity is *in*dependent of domain II in this context (Chapter 2, Fig. 10C). Recent reports of a TGFα-specific receptor conformation in cells would suggest that EGF- and TGFα-binding should be quite different, although there may be cellular-kinase-, and transmembrane-determinants that are missing in our *in vitro* binding studies (Scheck et al, 2012). Interestingly, it was recently observed that ligand-binding enthalpies for VEGFR-2 binding to VEGF-A, VEGF-C, and VEGF-E vary by up to 7kcal/mol, reflecting differences observed in ligand-VEGFR-2 crystal structures(Brozzo et al, 2012; Leppanen et al, 2011; Leppanen et al, 2010).

Given our interest in understanding whether different EGFR ligands engender distinct responses by forming unique receptor complexes via allosteric regulation, we sought to measure the thermodynamics of sEGFR-Fc-binding for Arg, Erg, and Epg. We reasoned that functionally unique receptor-ligand complexes might be manifested as unique patterns of ligand-binding

energies. For example, hydrogen bonds and salt bridges contribute to the enthalpy of ligand-binding, and solvation patterns contribute to the entropy of ligand-binding. Accordingly, if Arg, Erg, or Epg form receptor complexes that diverge substantially from those formed by TGFα and EGF, they should bind with a unique balance of enthalpy and entropy.

We performed ITC titrations of Arg, Erg, and Epg into sEGFR-Fc (Fig. 15 A-C, Table 5). The measured parameters are summarized in Table 5, and presented graphically in Fig. 15D. We found that Erg exhibits an intermediate affinity for sEGFR-Fc, in between the high-affinity binders EGF and TGFα, and Arg/Epg, which both bind with lower affinity. Broadly, each EGFR ligand we tested binds through an entropy-driven mechanism and exhibits highly disfavored enthalpic changes upon binding, in keeping with both EGF and TGFα and the VEGFR ligands. Furthermore, this pattern tracks with overall binding affinity; the low-affinity ligands Arg and Epg each exhibit an entropic driving force that is compromised by 6-9 kcal/mol compared to EGF and TGFα. The enthalpic penalty of binding for Arg and Epg, however, is reduced by 4-6 kcal/mol compared to EGF and TGFα, mitigating the effect of a diminished entropic driving force. The overall balance of entropic driving force and unfavorable enthalpies yields receptor-binding constants for Arg and Epg that are two orders of magnitude lower than those of EGF and TGFα. It must be noted, however, that the compensatory effects of enthalpy and entropy for these low- and high-affinity ligands argues that binding affinities alone would underestimate the divergence in the binding mode of Arg and Epg compared to EGF and TGFα. In particular, the observed difference in ligand-binding entropy of 6-9 kcal/mol for low- and high-affinity ligands is consistent with a dramatic change in buried surface area upon ligand-binding(Olsson et al, 2008). This entropic difference is consistent with a change of ~200-600 Å2 in the buried apolar surface area. EGF and TGFα each bury ~1500 Å2 total surface in published crystal structures. In addition, the enthalpic discrepancy for low- versus high-affinity ligands is consistent with a difference of 3-4 favorably hydrogen bonds. Clearly, these thermodynamic data are consistent with major functional and structural differences in Arg- and Epg-EGFR complexes compared to EGF- and TGFα-EGFR complexes.

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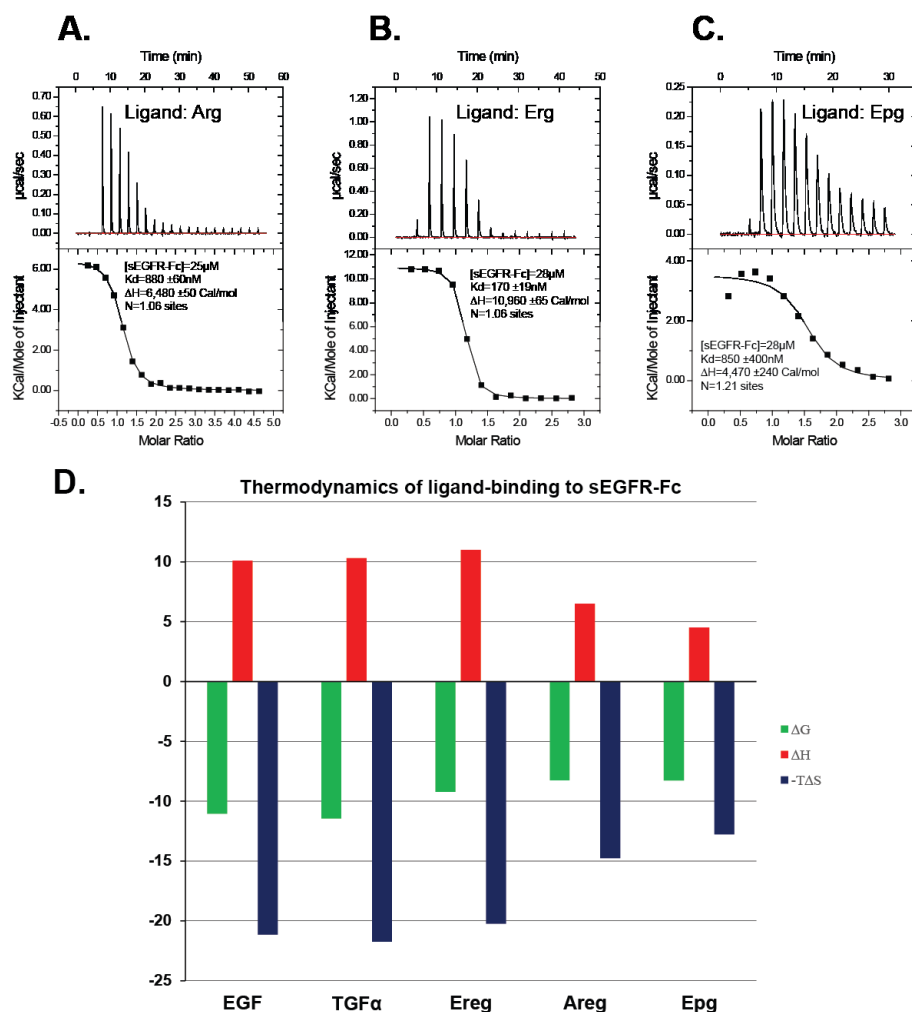
Figure 15*: A-C: ITC titrations of the ligands Arg (A.), Erg (B.), and Epg (C.) into 25-28µM sEGFR-Fc. All titrations were fit to a single-site binding model, and the calculated K_D and ΔH are specified on the plots. D. To visualize the enthalpic and entropic contributions of sEGFR-Fc binding for all the ligands tested at 25ºC, ΔG, ΔH, and ΔS values were plotted for this interaction. For EGF and TGFα, these values have been extracted from the plots in Fig. 8A-B.*

Table 5

EGFR variant	Ligand	$K_D^{Ligand-binding}$, nM	$\Delta S^{Ligand-binding}$, cal/mol/K	$\Delta H^{Ligand-binding}$, kcal/mol	N
sEGFR-Fc	Arg	880 ±60	49.5	+6.5 ±0.1	1.06
	Erg	170 ±19	67.8	+11.0 ±0.1	1.06
	Epg	850 ±400	42.8	+4.5 ±0.3	1.21
sEGFR-Fc$^{Y251A/R285S}$	Erg	2,740 ±300	38.7	+4.1 ±0.1	1.05
sEGFR^{501}	Epg	3,200 ±1,100	19.5	-1.7 ±0.1	1.03
sEGFR^{WT}	Epg	39,000 ±20,000	7.3	-3.9 ±2.8	0.67

Erg exhibits a unique pattern of ligand-binding enthalpy and entropy when compared to the high- and low-affinity ligands. It is similar to the high-affinity ligands when comparing the enthalpy and entropy in isolation. In that sense, one would predict it would mimic EGF and TGFα. However, the thermodynamic changes (small as they may be) in Erg-binding compared to EGF and TGFα do not compensate, as observed for Arg and Epg; instead, they each contribute additively, to strongly disfavor binding of Erg to sEGFR-Fc. Accordingly, the overall affinity of Erg is closer to that of the low-affinity ligands than the high-affinity ligands. Thus, where Arg and Epg are likely to exhibit significant structural differences in the receptor complexes they induce, our data suggests Erg induces a more subtle difference, but a potentially important one nonetheless.

Epigen- and Epiregulin-binding exhibit unique coupling to EGFR functional variants

The striking differences in thermodynamics for high-, low-, and intermediate-affinity EGFR ligand-binding to sEGFR-Fc is consistent with important functional differences for the ligands. These differences alone, however, do not provide functional insight. To test whether distinct EGFR ligands may induce functionally unique receptor signaling complexes, we extended our study of ligand-binding thermodynamics using functionally-relevant EGFR variants.

Given the importance of EGFR dimerization in receptor activation, we first sought to test how abrogation of the domain II dimerization interface affects ligand-binding. Recall from the previous chapter that the high-affinity ligands exhibit equivalent overall binding affinity, but a

slightly increased (less favorable) $\Delta H^{ligand\text{-}binding}$, upon mutation of the dimerization interface (Table 1). We performed ITC titrations of Epg, a low-affinity ligand, and Erg, an intermediate-affinity ligand that nonetheless mimics the thermodynamics of high-affinity ligands, into EGFR variants bearing either the wild-type dimerization interface or the Y251A/R285S double-mutation that abrogates receptor dimerization (Fig. 16 A-C). Epg exhibits an equivalent affinity for sEGFR[501] regardless of the status of the receptor dimerization interface (Fig. 16C, red). This same pattern holds for EGF and TGFα binding to sEGFR[WT]. In other words, receptor-mediated dimerization and ligand-binding are very weakly-coupled for Epg, EGF, and TGFα. In contrast, we found that Erg binding to sEGFR-Fc is highly sensitive to the receptor dimerization interface. While sEGFR-Fc binds Erg with a Kd of 170±19nM, sEGFR[Y251A/R285S]-Fc exhibits a 17.1-fold weaker affinity for Erg (SD=1.1; result of 2 independent experiments; Fig. 16C, green). These data argue that Erg binding is tightly-coupled to EGFR dimerization, in contrast to all other EGFR ligands tested.

A.

Time (min)
0 10 20 30 40 50

[sEGFR-Fc]=28µM
Kd=170 ±19nM
ΔH=10,960 ±65 Cal/mol
N=1.06 sites
Ligand: Erg

B.

Time (min)
0 10 20 30

[sEGFR-Ogiso-Fc]=17µM
Kd=2.74 ±0.3µM
ΔH=3,960 ±120 Cal/mol
N=1.05 sites
Ligand: Erg

C.

EGF
TGFalpha
Epigen
Epiregulin

Dimerization interface: WT   mut   WT   mut   WT   mut   WT   mut
EGFR variant used: sEGFR^WT   sEGFR^WT   sEGFR^GH   sEGFR-Fc

D.

Time (min)
0 10 20 30 40 50

[sEGFR-501]=43µM
Kd=3.2 ±1.1µM
ΔH=-1,670 ±76 Cal/mol
N=1.03 sites
Ligand: Epg

E.

Time (min)
0 10 20 30

[sEGFR-WT]=45µM
Kd=39 ±20µM
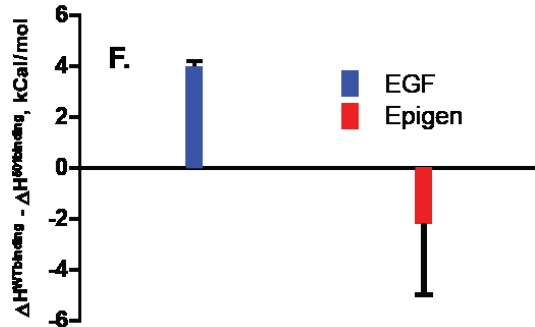ΔH=3,830 ±2,790 Cal/mol
N=0.666 sites
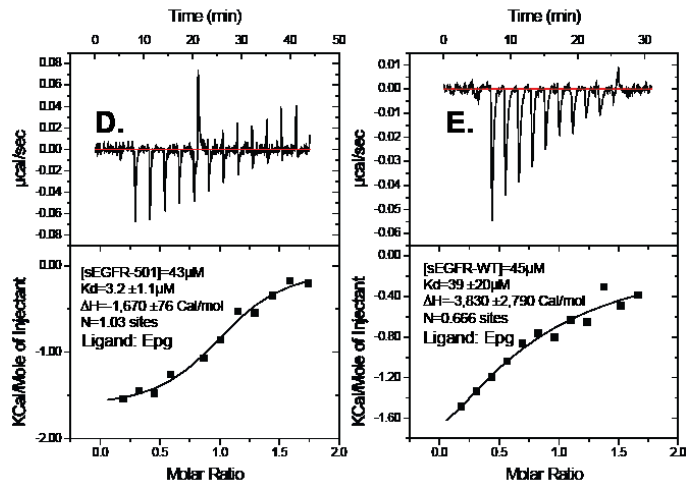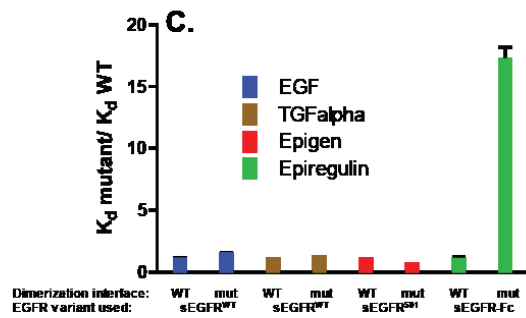Ligand: Epg

F.

EGF
Epigen

65

Figure 16: ITC titrations of Erg and Epg into sEGFR variants, to examine their thermodynamic coupling to EGFR functions compared to EGF. A: Erg titrated into 28μM sEGFR-Fc. B: Erg titrated into 17μM sEGFR-Fc$^{Y251A/R285S}$. C: The affinity dependence of EGFR ligands on domain II-mediated dimerization was compared by plotting the affinity ratios for matched EGFR proteins, with either wild-type or Y251A/R285S-mutated dimerization interfaces. For EGF (blue), the comparison was made with 2 replicates of ITC-derived values for binding to sEGFR^{WT} and sEGFR$^{Y251/R285S}$. For TGFα (South), the comparison was made with representative ITC-derived values for binding to sEGFR^{WT} and sEGFR$^{Y251/R285S}$. For Epg (red), the comparison was made with representative ITC-derived values for binding to sEGFR^{501} and sEGFR$^{501/Y251A/R285S}$. For Erg (green), the comparison was made with 2 replicates of ITC-derived values for binding of sEGFR-Fc and sEGFR-Fc$^{Y251A/R285S}$. Direct comparison for all ligands using the same EGFR proteins was not feasible due to technical constraints of ITC. D-E: ITC titrations of Epg into 43μM sEGFR^{501} (D.) and 45μM sEGFR^{WT} (E.). F: The difference between ΔH$^{ligand-binding}$ for sEGFR^{WT} and sEGFR^{501}, determined by ITC is plotted for EGF (blue) and Epg (red).

Because coupling of dimerization and ligand-binding for Epg appeared similar to EGF and TGFα despite its vastly different thermodynamics, we sought to test the coupling of Epg binding to another EGFR functional variant that is independent of dimerization. The sEGFR^{501} variant is a truncated version of sEGFR^{WT} that eliminates the tether interaction. As described in the previous chapter, this variant dramatically increases the binding affinity for EGF and TGFα by eliminating an intramolecular interaction that stabilizes the ligand-free form of EGFR. This has the effect of stabilizing EGF-binding by a favorable 3.9±0.3kcal/mol change in ΔH$^{ligand-binding}$ compared to sEGFR^{WT}(Fig. 16F, blue). This effect is consistent with the minimal model presented in Fig. 3, as long as domain IV contributes minimally to receptor dimerization (which we know to be true for EGF)(Dawson, 2005). In contrast, the effect on ΔH$^{ligand-binding}$ for Epg binding to sEGFR^{501} versus sEGFR^{WT} is strikingly different; Epg binding to sEGFR^{501} is actually

enthalpically dis-favored by 2.1±2.9kcal/mol compared to sEGFR^{WT} (Fig. 16D-E). The overall

discrepancy in $\Delta\Delta H^{501>WT}$ for Epg versus EGF is 6.0±3.2kcal/mol (Fig. 16F). Technical

challenges of the ITC experiment (due to low-affinity and a low $\Delta H^{ligand-binding}$) preclude a precise

determination of the Kd and ΔS for Epg binding to sEGFR^{WT}. Qualitatively, however, sEGFR^{501}

binds more tightly by about an order of magnitude (similar to EGF). The considerable difference

in $\Delta\Delta H^{501>WT}$ for Epg strongly argues that the minimal structural model depicted in Fig. does not

apply to Epg. So what is the functional effect of Epg binding to EGFR? Because Epg still signals

via EGFR (presumably through inducing homodimers, Fig. 14D), and abrogation of the tether

increases Epg affinity (implying that the tether interaction is broken upon ligand-binding, as for

other EGFR ligands), the best explanation is that Epg-induced EGFR homodimers are uniquely

dependent upon domain IV-mediated interactions.

Conclusions

It is widely assumed that all the EGFR ligands act through well-conserved allosteric

effects to activate EGFR. On the other hand, emerging evidence for ligand-specific effects on

receptor conformation and signaling output begs for a mechanistic explanation(Hobbs, 2002;

Scheck et al, 2012; Wilson et al, 2012a). I report here the first attempts (to my knowledge) to

broadly examine ligand-specific allosteric effects on EGFR. Compared to the prototypical ligand

EGF, we observed dramatic differences in the coupling of ligand-binding to dimerization (for Erg)

and the domain II-domain IV tether interaction (for Epg). In combination with the studies reported

in Chapter 2, we can thus begin to build a more complete model of allosterically-induced EGFR

signaling and make testable predictions of ligand-dependent receptor function.

We suggested in Chapter 2 that the preference of EGF for ErbB2:EGFR heterodimers (Li

et al, 2012) may explain the weak coupling of EGF binding to EGFR homodimerization. In order

to adaptively maintain a preference for ErbB2:EGFR heterodimers, EGF must adaptively maintain

an affinity for EGFR monomers that is at least comparable to that for EGFR homodimers.

Otherwise, EGF-induced EGFR homodimers would be the predominant complex formed when

ErbB2 and EGFR are both present. This sort of weak coupling between ligand-binding and homodimerization is also observed for the ligands TGFα and Epg. In contrast, the binding of Erg is tightly coupled to domain II-mediated EGFR dimerization. This observation suggests that Erg may be adapted to signal preferentially through EGFR homodimers, rather than through ErbB2:EGFR heterodimers. In agreement with this notion, it has been reported that Erg induces less ErbB2 transactivation compared to EGF when applied to cells overexpressing ErbB2(Komurasaki et al, 1997). Other reports have also shown Erg binds to and activates ErbB4 as well, and that Erg activation is dependent upon the presence of other ErbB receptors – consistent with ligand-specific preferences for certain ErbB signaling complexes(Jones, 1999; Riese et al, 1998). One important caveat for these studies is that they have all used bacterially-derived Erg proteins; bacterially-derived Erg binds EGFR with a ~10-fold lower affinity than the insect-derived Erg I have produced, and it lacks a beta-strand in the C-terminal region that is conserved in EGF, TGFα, and HB-EGF(Jones, 1999; Louie et al, 1997; Sato et al, 2003). Given these concerns, it will be important to re-evaluate patterns of Erg-induced ErbB activation with the insect cell-derived Erg we have created. Beyond simply inducing EGFR homodimers preferentially over ErbB2:EGFR heterodimers, it is also possible that Erg induces a distinct EGFR homodimer with unique signaling properties. The fact that EGFR homodimerization 'feeds back' to stabilize Erg-binding, but fails to stabilize EGF-, TGFα-, and Epg-binding, is indeed suggestive that Erg induces a unique EGFR homodimer conformation. Further experiments are needed to determine whether the Erg-EGFR homodimer complex exhibits unique signaling properties.

The ligand Epg appears to be insensitive to the EGFR domain II dimerization interface, like EGF and TGFα (and in contrast to Erg). On the other hand, it is highly sensitive to the presence of domain IV in a unique way, typified by a less-favorable $\Delta H^{\text{ligand-binding}}$ for sEGFR[501] compared to sEGFR[WT]. One possible explanation is that Epg induces an EGFR homodimer that uniquely exploits the domain IV dimerization interface. Prior work has suggested that the domain IV dimerization interface is dispensable for EGF-induced EGFR homodimerization (Dawson, 2005), but this may not be the case for Epg-induced EGFR dimerization. Domain IV-mediated

dimerization has been observed crystallographically, and it has been reported to play a key role in translating ligand-binding into receptor kinase activity (Arkhipov et al, 2013; Lu et al, 2010; Moriki et al, 2001). Accordingly, any ligand-specific difference in the domain IV dimeric interface could play an important role in modulating the EGFR signaling response in a ligand-specific way.

The observations we report here on the thermodynamics of EGFR ligand-binding will require further experiments to bridge the gap from biochemical observations to functional consequences in cells. Regardless, the striking and unexpected diversity in the thermodynamics of ligand-binding and in ligand sensitivity to receptor variants clearly argues that ligand-specific allosteric effects on EGFR do exist. This notion has been hinted at before, but our development of new reagents has allowed us to test this idea and begin to speculate about novel, ligand-specific functions(Wilson et al, 2009). Insight into the precise nature of ligand-specific signaling differences should allow the development of more effective ErbB-targeted therapeutics. For example, specific stimulation of ErbB2:ErbB4 heterodimers might abrogate trastuzumab-induced cardiomyopathy, and better functional mimics of Arg, Epg, and Erg might aid in vitro maturation (Richani et al) protocols for fertility treatments – where EGF alone is less effective than these ligands(Guglin et al, 2008; Richani et al, 2014).

CHAPTER 4: Perspectives and future directions

It will be useful to go back to the simplest possible concept of RTKs as transmembrane signaling modules when considering the data I have presented here. To date, RTKs are largely assumed to represent a discrete switch: they are either active or inactive, with no grey area in between. This notion contrasts with our understanding of another broad family of transmembrane signaling modules, the G-protein coupled receptors, or GPCRs. It has long been recognized that distinct GPCR ligands may induce discrete functional outcomes, and that the origin of these differences can be autonomous to the ligand:GPCR complex itself(Stephenson, 1997). Only more recently has the cellular context of a distinct ligand:GPCR signal come to the fore; it is now clear that a single GPCR ligand, acting through a single receptor, can simultaneously function as both an agonist and an antagonist(Urban et al, 2007a). The cellular interpretation of a GPCR ligand as agonist or antagonist can depend primarily on which specific output is measured(Urban et al, 2007b). For our purposes, this is a double-edged sword. On one hand, it is satisfying and compelling to uncover hidden complexity in the signaling response of a single transmembrane receptor. On the other hand, this knowledge argues against the broad utility of simple two-state models of receptor activity, and demands a much more complex model to understand (and to therapeutically exploit) all the known transmembrane receptors.

The conceptual parallels between GPCRs and RTKs here are striking. Like GPCRs, single RTKs can activate a multitude of signaling pathways simultaneously (Alroy & Yarden, 1997; Jones et al, 2006). Like GPCRs, RTK ligands have shown a variety of different thermodynamic mechanisms for binding to the same receptor (Borea et al, 1998; Brozzo et al, 2012). For GPCRs, the thermodynamics of ligand-binding has in some cases been highly correlated with the functional activity of the ligand(Borea et al, 1998). Such a ligand-dependent functional link between the thermodynamic mechanism of ligand-binding and the functional receptor output has not yet been established for RTKs. However, it has only very recently been recognized that RTK output may in fact be ligand-dependent(Wilson et al, 2009). The data I have presented above, not only showing that EGFR ligands bind through different thermodynamic mechanisms, but also that these ligands exhibit unique linkage to EGFR functional variants, argues quite clearly that

receptor-autonomous, ligand-dependent signaling outputs are feasible biophysically. In analogy to data I have presented for EGFR, others recently showed that the VEGFR-2 ligands VEGF-A, VEGF-C, and VEGF-E bind receptor with unique thermodynamics, and they each exhibit unique thermodynamic linkage to deletion of the membrane-proximal Ig domains D4-D7, which are required for VEGFR signaling(Brozzo et al, 2012). In combination, these data begin to suggest that receptor-autonomous, ligand-specific signaling responses may indeed be a general and unappreciated feature of RTK signaling. If this is true, it would have broad implications for understanding RTK function and for drug design.

The challenge in going forward will be to carefully test the biological relevance of the biophysical ligand differences we have observed. Three primary techniques will be exploited in the future to test the idea that EGFR ligands can induce unique responses by generating unique receptor complexes. First, cell-based assays in which all four ErbB receptors are combinatorially expressed will be stimulated with the ligands EGF, TGFα, Arg, Erg, and Epg. Because ErbB receptors signal by phosphorylating specific tyrosine residues on the receptor C-tail, we will measure receptor output at this level by using well-established, phosphorylation-site-specific antibodies for western blotting(Yang et al, 2006). This approach has an advantage over general phosphotyrosine detection, because it will be far more sensitive to signaling differences(Kim et al, 2012). Prior studies have suggested that Arg results in less phosphorylation at Y1045 of EGFR compared to EGF(Roepstorff et al, 2009; Willmarth et al, 2009). Based on the strong linkage I have observed between Erg binding and EGFR homodimerization, I hypothesize that Erg will show a relatively low level of ErbB2 phosphorylation compared to EGF when each ligand is applied to cells expressing EGFR and ErbB2, owing to a preference for EGFR homodimers.

Another approach will employ near-full length EGF receptor, which contains the ECR, the transmembrane domain, and the intracellular kinase domain, but no C-terminal tail. This protein has recently been purified in micelles and lipid nanodiscs, and shown to recapitulate EGF-dependent kinase activity(Mi et al, 2011; Qiu et al, 2009). I will add the different purified ligands

to this form of the receptor and perform kinase activity assays, to determine the linkage between ligand-binding and kinase activity for each ligand. Based on the observation that TGFα induces a unique conformation in the intracellular juxtamembrane region, which regulates kinase activity, I predict that I will observe different patterns of kinase activation for the different ligands I test(Scheck et al, 2012).

Finally, I will attempt to determine a high-resolution crystal structure of Arg, Erg, and Epg bound to different variants of EGFR, as well EGF bound to (mutated) monomeric EGFR. Preliminary crystals of an EGF:sEGFR$^{Y251A/R285S}$ variant have already been obtained. Based on the thermodynamics of ligand-binding, I predict that Arg, Erg, and Epg will exhibit slightly different ligand:receptor interactions compared to EGF and TGFα. Furthermore, I predict that Erg will induce a unique EGFR homodimer interface. Based on the poor thermodynamic linkage between EGFR homodimerization and EGF binding, I predict that an EGF:sEGFR$^{Y251A/R285S}$ complex will exhibit a domain II dimerization-arm conformation that is different from the conformation in the EGFR homodimer. Crystal structures of these complexes, should they differ from those observed for EGF- and TGFα-induced EGFR homodimers, will be used to design mutations to probe the functional implications of the observed conformations.

Chapter 5: Materials and methods

Reagents

EGF and TGFα were purchased from Millipore and R&D systems. Both sources gave identical results in ITC binding assays. All receptor proteins were expressed in Sf-9 cells utilizing a baculovirus system using the pFastbac (pFb) plasmid (Invitrogen). Construction of the tether-compromised and monomeric-EGFR variants has been previously described(Dawson, 2005). Glioblastoma mutations were introduced via Quikchange PCR. sEGFR-Fc was constructed by mutating pFb-sEGFR^{WT} to create an *FseI* restriction site at the c-terminus. cDNA encoding the Fc domain from human IgG1 (IMAGE clone 4575935) was purchased from Open Biosystems, and was used as a PCR template to create a fragment containing the Fc domain coding region flanked by *FseI* and *NotI* sites at the 5' and 3' ends, respectively. This fragment was cloned into the pFb-sEGFR^{WT} plasmid containing an *FseI* restriction site, yielding a plasmid coding for residues 1-645 of the EGFR protein, followed by 'AG' (introduced by the *FseI* site), and further followed by the 231 residue Fc domain, with a hexa-His tag at the C-terminus. sEGFR-ZIP was constructed by first amplifying a fragment encoding the 33-residue coiled-coil domain from yeast GCN4 by a series of 4 PCR reactions, each extending the length of the fragment at the 3' end. This fragment also contained 20 nucleotides at the 5' end that were complementary to the C-terminus of sEGFR, as well as a hexa-His tag and *NotI* site at the 3' end. This fragment was used as a primer to amplify (from a pFb-sEGFR^{WT} template) a fragment encoding sEGFR followed by the 33-residue coiled-coil domain and a His tag into pFb.

Protein purification

All EGFR receptor proteins were purified by the same procedure. Briefly, 4 days post-infection, conditioned media were concentrated to ~1l and diafiltered against 4l of buffer containing 20mM HEPES, pH 8.0, and 150mM NaCl (binding buffer). Diafiltered media was passed over NiNTA-agarose, and proteins were eluted by increasing concentrations of imidazole in binding buffer. Imidazole fractions containing receptor were dialyzed or buffer-exchanged via serial concentration into cation exchange buffer (20mM MES, pH 6.0, and 50 mM NaCl) and purified via

cation-exchange chromatography, followed by size-exclusion chromatography on a Superose 6 column in binding buffer.

Ligands were purified exactly as described for receptors, with a few exceptions. For Erg and Epg, hygromycin-selected stably-transfected S2 cell lines were created with a pMT vector encoding the expression cassette described in Fig. 14. Arg was produced using the pFastbac a baculovirus system as described for sEGFR above. Erg and Epg expression in S2 cells was induced by adding 500μM $CuSO_4$ to the culture medium, and media was harvested and diafiltered after 4 days of expression. Arg, Erg, and Epg were sequentially purified by NiNTA-agarose affinity chromatography, cation-exchange chromatography on an SO3 column, and size-exclusion chromatography on a Superose 12 column. For Erg, the buffer was adjusted via dialysis to 20mM HEPES, pH 7.5, and 70mM NaCl for the cation exchange step, while Arg and Epg were bound to the SO3 column in 20mM MES, pH 6.0, and 50mM NaCl. For Erg and Epg, a final purification step was added in which gel filtration fractions were passed first through a 50kDa-MWCO protein concentrator, and then through a 30kDa-MWCO protein concentrator. In each concentration step, approximately half the ligand was retained in the concentrator and half flowed through, while >90% of the contaminant proteins was retained. This step was important for accurate protein concentration determination by 280nm absorbance measurements, as the contaminant proteins absorbed strongly at 280nm.

Isothermal Titration Calorimetry

Receptor and ligand proteins were dialyzed overnight into ITC buffer, containing 20 mM HEPES, pH 8.0, 150 mM NaCl, and 3.4 mM EDTA. Receptor concentration in the cell was 10 μM unless otherwise specified, and ligand was injected at concentrations of 60-120 μM. All protein concentrations were determined by measuring absorbance of purified protein at 280nm and dividing by an extinction coefficient that was predicted from primary amino acid sequence. Generally, 20 ligand injections of 2 μL were performed for each receptor, and data from the first injection were always discarded to eliminate syringe leakage artifacts. For Erg and Epg titrations,

13 injections of 3 µL were performed to increase the signal-to-noise ratio for each injection. Ligand titrations into receptor-free ITC buffer were performed to determine the heat of ligand dilution when receptor was not fully saturated in a given experiment, and these heats were subtracted out from the receptor titration data. All experiments were performed at 25^0 C (except for those described in Fig. 9) in a Microcal ITC200 instrument. Data were fit to a single-site binding model in the Origin software package. All titrations were performed independently at least three times, and representative titrations are shown.

Fluorescence Anisotropy-based binding assays

EGF was labeled with Alexa-488 utilizing a TFP ester to label primary amines, according to the protocol provided with the Alexa Fluor 488 Protein Labeling Kit from Molecular Probes (Eugene, OR). After labeling, EGF was purified away from free dye by size-exclusion chromatography on a Superdex Peptide column in buffer containing 20mM HEPES, pH 8.0, plus 150 mM NaCl. Labeling efficiency was calculated by measuring the absorbance of purified, labeled EGF at both 280 nm and 490 nm, and assuming an extinction coefficient of 18,825 $cm^{-1}M^{-1}$ at 280 nm (as predicted from the primary sequence of EGF) and 71,000 at 490 nm (for the Alexa-488 dye). 10 nM (for sEGFR-Fc and sEGFR-Zip) or 60 nM (for sEGFR^{WT}) EGF488 was incubated with varied amounts of receptor protein for 30 minutes at RT in buffer containing 20mM HEPES, pH 8.0, and 150mM NaCl. Fluorescence polarization (FP) measurements were taken on a Beacon instrument at 20º C. FP values were converted to anisotropy, and binding curves were derived by assuming that the maximal anisotropy response corresponded to [EGFfree]=0, while the anisotropy in the absence of receptor corresponded to [EGFfree]=[EGFtotal]. These curves were fit to binding models using the Graphpad Prism software. sEGFR-Fc and sEGFR^{WT} binding data were fit to simple single-site binding models, while sEGFR-Zip binding data were fit to a Hill model with a Hill coefficient of ~1.9, presumably reflecting the presence of sEGFR-Zip monomers at very low receptor concentrations.

Electron microscopy

Receptor samples at a concentration of 2 µg/ml in binding buffer were applied to glow-discharged carbon grids and stained with 0.75% uranyl formate. Images were collected on a Tecnai T12 microscope at 67,000x magnification and operating at 120 keV. For the EGF:sEGFR-Fc complex, 755 individual particles, manually picked from 150 images using the EMAN software package (Ludtke et al, 1999), were grouped into 10 classes by a reference-free alignment procedure in Spider (Frank et al, 1996). For the sEGFR-Fc protein alone, 2,566 particles from 673 images were grouped into 20 classes by the same reference-free alignment procedure.

Small-angle x-ray scattering

SAXS protein samples were prepared at concentrations of 10-20 µM in binding buffer, and 40 minute exposures at 4°C were performed on a Rigaku SMAX3000 instrument, using a Rigaku 007HF rotating anode source and a Rigaku 300mm wire grid ASM DTR 200 detector. Scattering data were radially-averaged and reduced to two-dimensional plots using the SAXSgui software, and intensity data from buffer exposures was then subtracted out. Radii of gyration were determined by Guinier plots using the Primus software package. The maximal interatomic distance (Dmax) was obtained by examining p(r) curves generated by the Gnome software package. Briefly, for each scattering dataset, p(r) curves were calculated for a range of Dmax from 100 to 250 Å, in 5Å increments. Dmax was determined by finding the value that gave the best fit to the experimental scattering data.

Analytical ultracentrifugation

Receptor protein samples at a concentration of either 10 µM (sEGFR^{WT}) or 5 µM (sEGFR^{R84K}, sEGFR^{A265V}, and sEGFR^{A265D}) were loaded into 6-hole sample cells for sedimentation equilibrium analytical ultracentrifugation analysis with a Beckman XL-A instrument at rotor speeds of 9,000 and 12,000 rpm. For conditions with TGFα, the ligand was present in a 1.2-fold excess over receptor protein. Data were analyzed using Sedfit and Sedphat, as well as simple log plots of absorbance intensity data, as previously described (Ferguson, 2000). For log plots, a constant

value was added to each data point within a single sample to align the log plots to the origin; this procedure does not affect the fit, which depends only on the slope of the line.

Cell stimulation

Cell lines (either A431 or MDA-MB-468) were cultured in DMEM containing 10%FBS, and 100u/ml of penicillin and streptomycin. Cells were split into 10mm culture dishes at a density of ~50%. Once cells reached 80-90% confluence, they were switched to serum-free media and incubated overnight. Cells were then cooled on ice for 30 minutes and washed with stimulation buffer (cold DMEM containing 3% BSA). Cells were stimulated by adding 5ml of ligand, at the specified concentration, in stimulation buffer. As a positive control, carrier-free EGF from Millipore was used. After 10 minutes, the cells were immediately rinsed with stimulation buffer, and harvested in 1mL RIPA buffer (containing PMSF, Leupeptin, Aprotinin, NaF, Vanadate, and Molybdate to inhibit proteases and phosphatases) using a cell scraper. Cells were lysed for 30 minutes at 4^0C on a Nutator. Cell lysates were clarified by spinning at 15,000 RPM at 4^0C in a benchtop centrifuge, and the supernatant was used for analysis by western blotting. Alternatively, 500 µl was incubated for 1 hour with 50 µl of Concanavalin-A 4B sepharose in RIPA (GE) at 4^0C to enrich for glycosylated proteins. Proteins were then eluted by boiling in reducing PAGE sample buffer containing 1mM EDTA for analysis by western blotting.

Western blotting

Blotting samples were run on a 7.5% PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked for 1 hour at 4^0C with PBS+3% BSA. PY20 antibody (Jaiswal et al) was added at 1µg/ml, and incubated overnight at 4^0C. Membrane was washed 3x in 5ml of PBS, and HRP-linked anti-Mouse antibody was added and incubated for 1 hour at room temperature. The blots were imaged using the SuperSignal ECL kit (Pierce).

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