An Open-and-Shut Case?
Recent Insights into the Activation
of EGF/ErbB Receptors

Review

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Recent crystallographic studies have provided significant new insight into how receptor tyrosine kinases from the EGF receptor or ErbB family are regulated by their growth factor ligands. EGF receptor dimerization is mediated by a unique dimerization arm, which becomes exposed only after a dramatic domain rearrangement is promoted by growth factor binding. ErbB2, a family member that has no ligand, has its dimerization arm constitutively exposed, and this explains several of its unique properties. We outline a mechanistic view of ErbB receptor homo- and hetero-dimerization, which suggests new approaches for interfering with these processes when they are implicated in human cancers.

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Since its discovery more than 40 years ago (Cohen, 1960), epidermal growth factor (EGF) has provided numerous puzzles for biologists, biochemists, physicists, and oncologists (Jorissen et al., 2003; Schlessinger, 2002). In the past year, major advances in understanding EGF action have come from crystallographic studies of extracellular regions from three members of the EGF receptor (EGFR) or ErbB family: namely EGFR itself, ErbB2 (HER2/Neu), and ErbB3 (HER3) (see Table 1). The structures have yielded several surprises. A dramatic conformational transition was shown to occur upon ligand binding; an unprecedented (entirely receptor-mediated) mode of dimerization was identified; and an unexpected apparently "preactivated" state was defined for the ErbB2 monomer. By combining the information gained from the recent structural studies, we have been able to develop models for the allosteric regulation of EGFR family members. These models have greatly improved our understanding of ErbB receptor signaling and have created opportunities for the design of new anticancer agents.

EGFR was the first cell-surface receptor to be linked directly to cancer when Stanley Cohen and colleagues described the downregulation of EGFR in fibroblasts infected with oncogenic viruses (de Larco and Todaro, 1978). The findings that EGFR is a ligand-dependent tyrosine kinase (Ushiro and Cohen, 1980) and that the product of the v-erbB oncogene from avian erythroblastosis virus is a truncated form of EGFR (Downward et al., 1984), revolutionized both growth factor and cancer biology. We now know that many carcinomas are promoted by EGFR activation, which can result from mutation of the receptor (Humphrey et al., 1990; Jungbluth et al., 2003), its overexpression (Arteaga, 2002), or from EGFR stimulation through autocrine loops (Sizeland and Burgess, 1992).

ErbB Receptors Are Activated
by Ligand-Induced Dimerization

It is well established that growth factor binding to the extracellular region of EGFR promotes dimerization of the monomeric receptor and increases the tyrosine kinase activity of its intracellular domain (Schlessinger, 2000). Receptor molecules in the ligand-induced EGFR dimer become tyrosine autophosphorylated in *trans*. The resulting phosphotyrosines recruit the SH2 domains of multiple downstream signaling molecules, thus initiating an array of intracellular signaling pathways (Schlessinger, 2000). There has been considerable confusion about the precise mechanism of EGFR kinase activation by extracellular ligand binding. Ligand availability is clearly paramount in controlling the biological activity

Table 1. Structural Analysis of ErbB Family Extracellular Regions

Family Member (pdb ID)	Fragment	Ligand	Receptor Conformation	Reference
hEGFR (1mox)	1–501	TGF-α	extended	Garrett et al., 2002
hEGFR (1ivo)	1–619	EGF	extended	Ogiso et al., 2002
hEGFR (1nql)	1–621	EGF	tethered	Ferguson et al., 2003
hErbB2	1–509	N/A	extended	Garrett et al., 2003
hErbB2 (1n8y)	1–631	N/A	extended	Cho et al., 2003
rErbB2 (1n8z)	1–631	N/A	extended	Cho et al., 2003
hErbB3 (1m6b)	1–621	–	tethered	Cho et al., 2002

N/A, not applicable.

of EGFR family members. However, receptor display, dynamics, affinity, and competency also play important regulatory roles. EGFR appears to exist in two different affinity classes at the cell surface (Ullrich and Schlessinger, 1990), with 2%–5% of receptors binding EGF with high affinity ($K_D < 0.1$ nM) and 92%–95% binding with lower affinity (K_D 6–12 nM). The affinity classes are thought to represent different receptor conformations and/or oligomers. In addition to understanding the mechanism of ligand-induced dimerization per se, explaining the functional and structural characteristics of the different EGFR affinity states is an important goal. The recently determined structures of ErbB receptor extracellular regions in different activation states provide significant insight into both of these issues.

Growth Factor Ligands that Regulate the Four ErbB Receptors

EGFR is regulated by a family of at least seven distinct peptide ligands (Groenen et al., 1994; Harris et al., 2003), including EGF, transforming growth factor-α (TGF-α), amphiregulin, betacellulin, epigen, epiregulin, and heparin binding EGF-like growth factor (HB-EGF). ErbB2 has no known direct activating ligand (Citri et al., 2003), while ErbB3 and/or ErbB4 function as receptors for the four known neuregulins (NRGs) (Falls, 2003). All EGFR ligands are expressed as type I integral membrane proteins (Harris et al., 2003) and are proteolytically processed to yield the 49–85 amino acid mature growth factor that consists largely of the EGF-like domain (Harris et al., 2003). The bioactive core of mature NRG isoforms is also their EGF-like domain (Falls, 2003).

Domain Organization and Relationships with the ErbB Receptor Family

The four ErbB receptors are closely related single-chain modular glycoproteins with an extracellular ligand binding region (~620 residues), a single transmembrane domain (~23 residues), and an intracellular tyrosine kinase domain (~260 residues) that is flanked by juxtamembrane (~40 residues) and C-terminal (~232 residues) regulatory regions (Figure 1). It is intriguing that this family includes an orphan receptor that nonetheless has robust tyrosine kinase activity (ErbB2) and a demonstrated NRG receptor (ErbB3) that lacks tyrosine kinase activity (Guy et al., 1994). Each ErbB receptor is thought to have a distinct physiological role, which can be modified by ligand-induced formation of ErbB receptor heterooligomers that are capable of generating unique

signaling responses (Holbro et al., 2003; Yarden and Sliwkowski, 2001). Thus, the signaling characteristics of the 4 ErbB receptors are strongly interdependent.

The extracellular region of ErbB receptors is quite heavily glycosylated. In the case of EGFR, 9 of 11 potential glycosylation sites are utilized (Zhen et al., 2003), and carbohydrate accounts for an estimated 20% of the
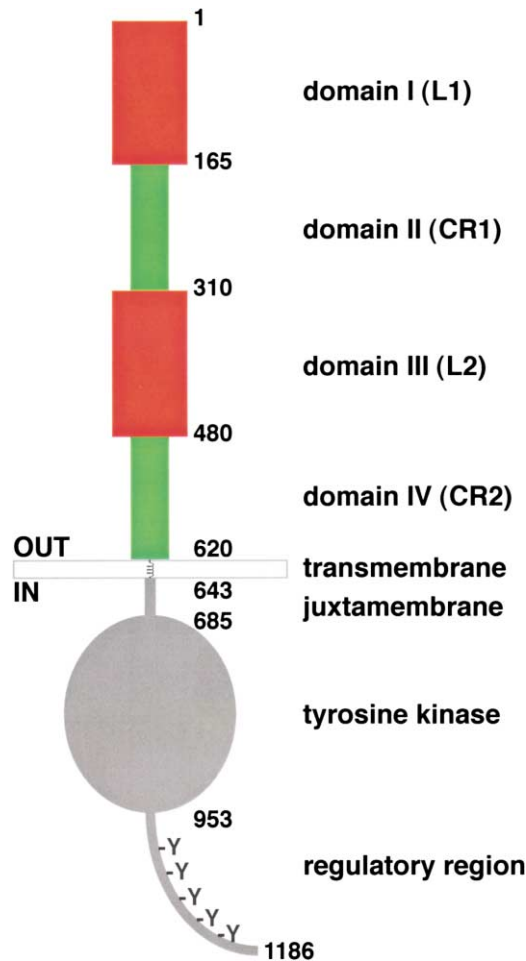

Figure 1. Domain Organization of ErbB Receptors

Throughout this report, the domains are referred to using the I, II, III, IV nomenclature (Lax et al., 1988b). An alternative nomenclature using domain names L1, CR1, L2, CR2 (Ward et al., 1995) is also used in the literature. Residue numbers for domain boundaries are for EGFR.
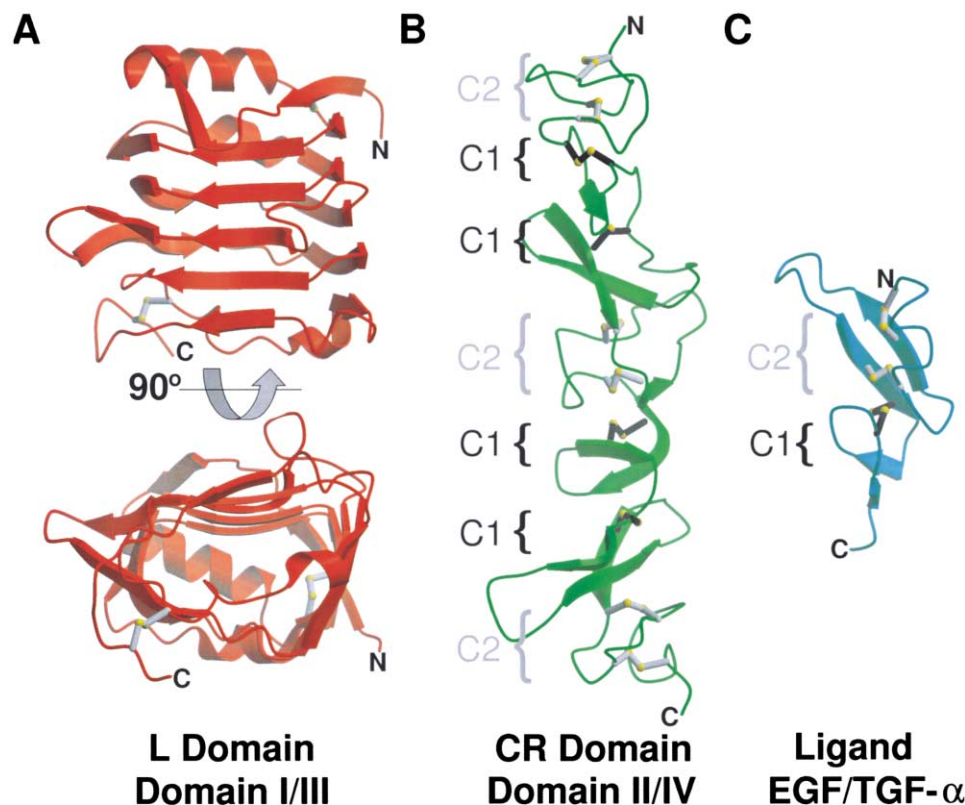
Figure 2. Structural Characteristics of Individual Domain Types

(A) Domain I (L1) from sEGFR (Garrett et al., 2002) is shown in two orthogonal orientations as a representative β helix or solenoid domain.

(B) Domain IV (CR2) from sEGFR (Ferguson et al., 2003) is shown as a representative cysteine-rich domain. The ladder of disulfide bonds can be seen clearly. The order of disulfide-bonded modules in this domain is C2-C1-C1-C2-C1-C1-C2, as marked beside the domain. Disulfides in C2 modules are gray; those in C1 modules are black.

(C) EGF from the sEGFR•EGF complex (Ogiso et al., 2002) is shown as a representative ligand molecule. EGF contains two small (2-stranded) β sheets. As labeled here, it can be viewed as containing two modules: one related to a C2 module and the other to a C1 module.

molecular mass (Lax et al., 1990). Four distinct protein domains, of two different types, constitute the ErbB receptor extracellular regions. There are two homologous large (L) domains, and two cysteine-rich (CR) domains, which occur in the order L1-CR1-L2-CR2 (Ward et al., 1995). These four domains have alternatively been named L1-S1-L2-S2 (Bajaj et al., 1987), and I-II-III-IV (Lax et al., 1988b). For the sake of clarity, and to assist readers outside the EGFR field, we use only the I-II-III-IV nomenclature in this review (see Figure 1). Related domains are also found in the extracellular regions of receptor tyrosine kinases from the insulin receptor family (Ward and Garrett, 2001), which are disulfide-linked dimers (by contrast with ErbB receptors), and bind to quite different sets of ligands. The L domains (domains I and III in ErbB receptors) are members of the leucine rich repeat (LRR) family. The CR domains (domains II and IV in ErbB receptors) were predicted to contain multiple small disulfide-bonded modules similar to those in laminin (Figure 2). These modules are defined either by a single disulfide bond (a C1 module) or by two intertwined disulfides that link side chains in the pattern Cys1-Cys3 and Cys2-Cys4 (a C2 module) (Ward et al., 1995). This prediction has been confirmed experimentally by disulfide bond mapping (Abe et al., 1998)

and by X-ray crystal structures (Table 1). Domain II of ErbB receptors contains 8 disulfide-bonded modules in the order C2-C2-C2-C1-C1-C1-C1-C1. Domain IV contains 7 modules, in the order C2-C1-C1-C2-C1-C1-C2. For comparison, a single laminin repeat contains 3 modules in the order C2-C1-C1 (Adams et al., 2000). The first C2 module of each ErbB receptor CR domain has an intimate relationship with its preceding L domain, and appears to be required for proper L-domain folding. Intriguingly, the ≈50 amino acid EGF-like domain of ErbB ligands, characterized by a distinct pattern of 6 disulfide-bonded cysteines, can also be thought of as containing two related disulfide-bonded modules in the order C2-C1 (Figure 2C) (Abe et al., 1998; Ward et al., 2001).

Structures of ErbB Receptor Extracellular Regions

The recently described crystal structures of ErbB receptor extracellular regions (Table 1) showed that their L and CR domains adopt the right-handed β helix (or "solenoid") and laminin-like folds, respectively, which were previously seen in the extracellular region of the insulin-like growth factor-1 receptor (IGF-1R) (Garrett et al., 1998; Ward et al., 2001). As shown in Figure 2A, domains I and III of ErbB receptors form a six-turn right-handed
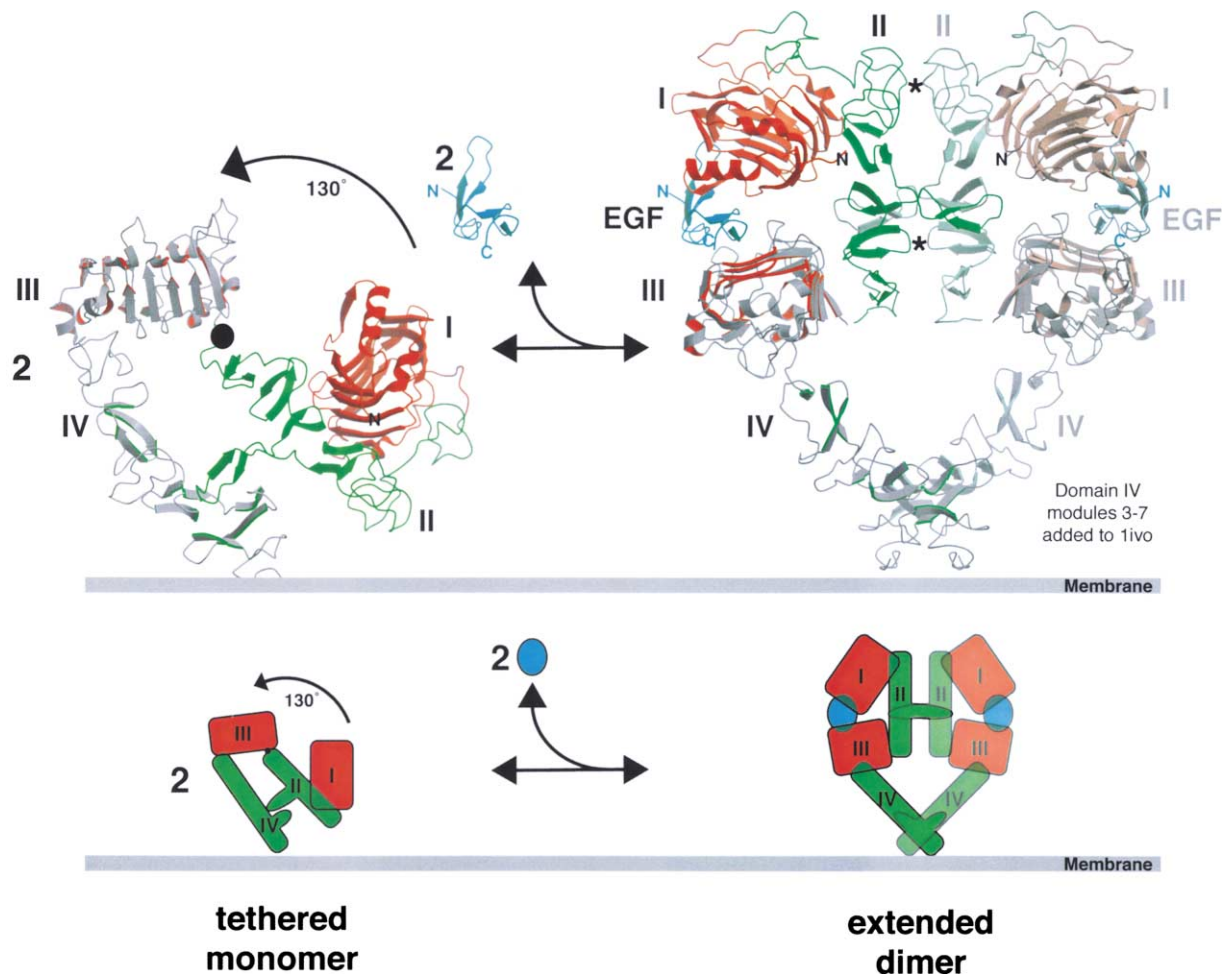
Figure 3. Schematic of Ligand-Induced Conformational Changes in sEGFR and Dimerization

A transition between two sEGFR structures is shown in both ribbons and cartoon representation. The unactivated (tethered) sEGFR structure (Ferguson et al., 2003) is shown on the left. A model of the EGF-induced dimer is shown on the right. This model uses the coordinates of Ogiso et al. (2002), which lacked 5 of the 7 disulfide-bonded modules of domain IV. We have added the missing modules of domain IV using the structure of unactivated sEGFR, and assuming that the domain III/IV relationship in sEGFR is unaltered upon ligand binding.

L domains in the receptor (domains I and III) are colored red, and CR domains (domains II and IV) are green. Ligand is colored cyan. Domains I and III are distinguished from one another by the addition of gray to the outer surfaces of strands and helices. The two subunits in the dimer are distinguished by the fogging of the right-hand dimerization partner. Individual domains are labeled. The mutual "hooking" of the two domain II dimerization arms across the dimer interface can be observed in the center of the structure. The additional domain II contacts across the interface, at module 2 (2nd C2 module) and module 6 (3rd C1 module), are marked with asterisks. The speculated position of the plasma membrane is depicted as a gray bar.

EGF binding is proposed to induce a 130° rotation of a rigid body containing domains I and II, about the axis represented by a filled black circle (at the domain II/III junction). This exposes the dimerization arm and allows dimerization of sEGFR, as depicted on the right.

β helix that is capped at each end by an α helix and a disulfide bond. Domains II and IV contain 8 and 7 disulfide-bonded modules, respectively, with the predicted disulfide bond arrangements (Ward et al., 1995). It is remarkable that in less than a year we have progressed from having no detailed structural information on the EGFR family to having 7 different crystal structures, with distinct arrangements of the receptor domains, that represent snapshots of both inactive and activated configurations (Table 1). This wealth of data has revolutionized our view of how ErbB receptors are regulated, and (as discussed in detail below) provides satisfying explanations for the unique biological properties of the orphan receptor ErbB2.

Paradigm of Receptor Dimerization Mediated by Bivalent Ligand Binding

It was previously suggested that EGF induces EGFR dimerization by binding simultaneously to two receptor molecules, and thus "crosslinking" them into a dimer (Gullick, 1994; Lemmon et al., 1997; Tzahar et al., 1997). This hypothesis followed largely from the paradigm established for human growth hormone (hGH), which forms a 1:2 complex with the extracellular regions of two hGH receptor molecules (de Vos et al., 1992). Structural studies of several other RTK dimers located a bivalent (usually dimeric) ligand species at the receptor-receptor interface where it directly mediates dimerization. This arrangement has been seen for portions of the vascular

endothelial growth factor (VEGF) receptor Flt-1 (Wiesmann et al., 1997), the nerve growth factor (NGF) receptor TrkA (Wiesmann et al., 1999), and EphB2 (Himanen et al., 2001). In the case of EGFR, it was well established that two ligand molecules (which are monomeric when studied alone) are bound in an EGF-induced dimer of the EGFR extracellular region (Domagala et al., 2000; Lemmon et al., 1997; Odaka et al., 1997). This stoichiometry is consistent both with mechanisms where EGF binding induces conformational changes that promote receptor-mediated EGFR dimerization, and with mechanisms in which two ligand molecules span the dimer interface to mediate receptor dimerization directly (Lemmon et al., 1997).

EGFR Dimerization Is Mediated Exclusively by Receptor Contacts

Contrary to most expectations, the crystal structures of ligand-bound sEGFR showed that dimerization is entirely receptor mediated (Garrett et al., 2002; Ogiso et al., 2002). The structures confirmed that two individual ligand molecules are present in the dimer. However, the two bound TGF-α (Garrett et al., 2002) or EGF (Ogiso et al., 2002) molecules could hardly be further from the dimer interface (Figure 3, right). Almost all receptor-receptor contacts observed in the crystal structures are mediated by domain II. At the center of the dimer interface is a prominent loop (residues 242–259 of EGFR) that extends from the second C1 module (module 5) of each domain II and reaches across the interface to interact primarily with domain II of its dimerization partner (Figure 3). This domain II loop, which is specific to ErbB receptors, has been termed the "dimerization arm" (Ogiso et al., 2002), and deletions or mutations in this region completely prevent ligand-induced EGFR activation (Garrett et al., 2002; Ogiso et al., 2002). In addition to the dimerization arm contacts, there are two smaller interaction sites in the dimer that involve side chains from the second and the sixth disulfide-bonded modules of domain II, and are marked with asterisks in the right-hand side of Figure 3 (Garrett et al., 2002). Moreover, it is possible that the dimer interface extends into domain IV. It has been reported that peptides modeled on disulfide-bonded modules 6 and 7 of domain IV can disrupt ErbB receptor homo- and heterodimerization (Berezov et al., 2002), and domain IV mutations can impair the ability of ligand to bind and induce tyrosine phosphorylation of EGFR (Saxon and Lee, 1999). The position of domain IV could not be defined in the initial sEGFR dimer structures, because most of this domain was absent from the crystallized protein (Garrett et al., 2002) or was not well ordered (Ogiso et al., 2002). In the right-hand side of Figure 3, we have added the rest of domain IV to the dimer model, assuming that the relationship between domains III and IV in unactivated sEGFR is preserved in the active dimer. This places domain IV of the two receptors very close to one another in the dimer, suggesting that they may directly interact. In very recent studies, most of domain IV in the sEGFR•EGF dimer complex has now been traced (Ishitani and Yokoyama, unpublished data), and occupies the position shown in Figure 3. The two receptor molecules approach one another very closely toward the C terminus of domain IV, although a well-defined, tight interface is not observed.

Ligand Binding to an EGFR Dimer

Although EGF and TGF-α clearly do not span the dimer interface, each ligand simultaneously contacts two separate binding surfaces in the same EGFR molecule. The two ligand binding surfaces are contributed by the β helix or solenoid domains I and III, as indicated by earlier affinity crosslinking studies (Lax et al., 1988a; Summerfield et al., 1996; Woltjer et al., 1992; Wu et al., 1990), domain-swapping experiments (Lax et al., 1989), and analysis of proteolytic EGFR fragments (Kohda et al., 1993). The bound EGF or TGF-α molecule resembles a wedge between domains I and III (Figure 3).

The types of interaction made with ligand are quite different for domains I and III (Garrett et al., 2002; Ogiso et al., 2002). While domain I contacts are primarily backbone mediated, ligand binding to domain III is primarily side chain mediated and involves many of the ligand side chains found to be important in earlier mutational studies (reviewed by Groenen et al., 1994). In particular, a highly conserved arginine in the ligand (R42 in TGF-α, R41 in EGF) interacts with a conserved aspartate in EGFR, and a critical aliphatic ligand side chain (L48 in TGF-α, L47 in EGF) projects into a well-defined hydrophobic pocket on the domain III surface.

Structures of Unactivated ErbB3 and EGFR Suggest an "Autoinhibited" Monomer

The sEGFR dimer structures provide a clear view of interactions that stabilize the receptor dimer, but do not explain how ligand binding actually drives receptor dimerization. By binding simultaneously to both domains I and III, the ligand may alter the relative orientations of these two domains. This could in turn change the conformation of the intervening domain II, so that its ability to dimerize is enhanced (Garrett et al., 2002). As mentioned below, domain II conformational changes of this sort almost certainly play an important role in ligand-induced EGFR dimerization. However, an additional layer of regulation, involving an unexpected role for domain IV, was suggested by crystal structures of both unliganded sErbB3 (Cho and Leahy, 2002) and an unactivated form of sEGFR (with EGF bound only to domain I) (Ferguson et al., 2003).

All four domains in the extracellular region were well defined in these unactivated structures. The relationship between domains I and II is essentially identical to that seen in IGF-1R and in the activated sEGFR dimer, implying that ligand binding does not greatly influence the relative orientation of these two domains. By contrast, the relationship between domains II and III differs dramatically in the activated and unactivated structures (Figure 3). A direct intramolecular interaction between cysteine-rich domains II and IV restrains the domain II/III relationship that characterizes the unactivated configuration. This interdomain "tether" is stabilized by essentially identical interactions between the two cysteine-rich domains (II and IV) in inactive sErbB3 (Cho and Leahy, 2002) and sEGFR (Ferguson et al., 2003). Interdomain hydrogen bonds are made by four or five residues that are well conserved in EGFR, ErbB3, and ErbB4 but not in ErbB2. Some of these residues are in the domain II dimerization arm and appear to have dual roles—contributing alternatively to the intramolecular domain

II/IV tether in the inactive receptor or to dimerization in the activated receptor. Most intriguingly, the intramolecular domain II/IV tether precisely buries the dimerization arm of domain II against domain IV, so that the tethered configurations of sErbB3 and sEGFR cannot dimerize in the manner shown in Figure 3, and thus appear to be autoinhibited (Cho and Leahy, 2002; Ferguson et al., 2003; Schlessinger, 2003). Moreover, the two ligand binding surfaces on domain I and III are too far apart in the tethered configuration for a single ligand to bind to both simultaneously. Consequently, the tethered configuration can only form low-affinity interactions with ligand, using just one of its ligand binding surfaces at a time.

Ligand-Induced Activation of EGFR

A substantial domain rearrangement is required to switch between the unactivated and activated configurations of sEGFR (Figure 3). For a single ligand molecule to contact binding surfaces on both domains I and III simultaneously, these domains must be drawn toward one another, requiring the receptor to become "extended" as shown in going from left to right in Figure 3. Starting from the tethered configuration, and holding domain III in place, simultaneous binding of ligand to both domains I and III requires a $\sim$130° counterclockwise rotation of the rigid domain I/domain II pair about the black circle drawn in Figure 3, in addition to a $\sim$20 Å translation into the page. This domain rearrangement gives rise to the extended configuration, and breaks the intramolecular domain II/IV tether so that the dimerization arm is exposed for participation in *inter-*, rather than *intra*molecular interactions. Thus, only the extended configuration of sEGFR is capable of both high-affinity ligand binding and efficient dimerization.

Although the crystal structures provide snapshots of tethered and extended (dimeric) configurations of ErbB receptors, they do not explain the mechanism of activation. It remains unclear whether ligand binding actively induces conformational changes required for dimerization, although the fact that no significant (static) structural differences are found between the EGF-bound and unliganded states of domain III (Ferguson et al., 2003; Ogiso et al., 2002) argues against this possibility. We therefore currently favor a model in which ligand binding traps the extended configuration (in a monomeric or dimeric state) from the ensemble of conformations sampled by a flexible and dynamic receptor molecule. It should be noted, though, that such conformational variability has not been demonstrated experimentally. We estimate the strength of the intramolecular domain II/IV tether in EGFR to be $\sim$1–2 kcal/mole. Disrupting the tether (by mutation or domain IV deletion) increases ligand binding energy by 1–2 kcal/mol (Elleman et al., 2001; Ferguson et al., 2003), which we ascribe to loss of the tether as an energetic barrier to domain rearrangement. Assuming free equilibration between tethered and untethered configurations, this estimate suggests that at any given time $\sim$95% of sEGFR molecules will be tethered, and the remaining 5% will not. Since EGF will bind most strongly to the relatively rare extended form—because it can simultaneously contact binding sites on domains I and III—the presence of li-

gand will drive the equilibrium shown in Figure 3 to the right, trapping receptor molecules in the extended state that can dimerize. EGFR dimerization will thus be promoted, leading to receptor activation.

While exposure of the dimerization arm is clearly necessary, it is not sufficient on its own to drive EGFR dimerization. Indeed, a truncated form of sEGFR that lacks most of domain IV does not dimerize without ligand (Elleman et al., 2001; Garrett et al., 2002), despite being unable to form the intramolecular tether and therefore presumably having its dimerization arm exposed. It is possible that the dimerization arm must cooperate with additional contact sites in order to drive efficient receptor association. Two such sites, in modules 2 and 6 of domain II, are marked with asterisks in Figure 3, as mentioned above. Binding of ligand to domains I and III appears to promote (or trap) a spine-like bend in domain II that may bring these two contact sites and the dimerization arm into the appropriate register for them all to cooperate with one another at the dimer interface.

The Intracellular Tyrosine Kinase Domain of EGFR Lacks Autoinhibitory Interactions

While the extracellular region of EGFR is distinguished by autoinhibitory intramolecular interactions, a recent structure of its intracellular tyrosine kinase domain is notable for an *absence* of autoinhibitory interactions (Stamos et al., 2002). The kinase domains of most RTKs are catalytically inactive until ligand-induced dimerization causes them to become autophosphorylated within the activation loop (Hubbard and Till, 2000). Without phosphorylation, the activation loop normally adopts a conformation that inhibits substrate binding and holds critical catalytic side chains in a nonfunctional arrangement (Huse and Kuriyan, 2002). Activation loop phosphorylation normally induces significant conformational changes that remove the inhibition of substrate binding and place the catalytic groups ideally for phosphotransfer. The tyrosine kinase domain of EGFR is highly unusual in not requiring such activation loop phosphorylation to promote its activity (Gotoh et al., 1992).

The crystal structure of the unphosphorylated EGFR kinase domain (Stamos et al., 2002) suggests one explanation for this distinction. Even without phosphorylation, the EGFR activation loop adopts the conformation normally observed only in phosphorylated and activated kinases (Figure 4). An unmodified tyrosine side chain (Y845) in the EGFR activation loop occupies almost exactly the same position as a phosphotyrosine (pY1163) in the activated insulin receptor kinase. Nearby acidic side chains in EGFR are proposed to hold the Y845 side chain in this position, perhaps replacing the need for its phosphorylation. The most straightforward interpretation of this finding is that the activation loop of the EGFR kinase domain constitutively adopts the "activated" conformation, and is constitutively active.

There are several caveats to this simple interpretation. For example, it is possible that the activated conformation is not adopted spontaneously in solution but is promoted by interactions between kinase domains within the crystal. Since the EGFR activation loop does not participate in any crystal contacts, this could only occur though indirect, allosteric, effects. Several pieces

A-loop
EGFRK
pY-IRK
IRK

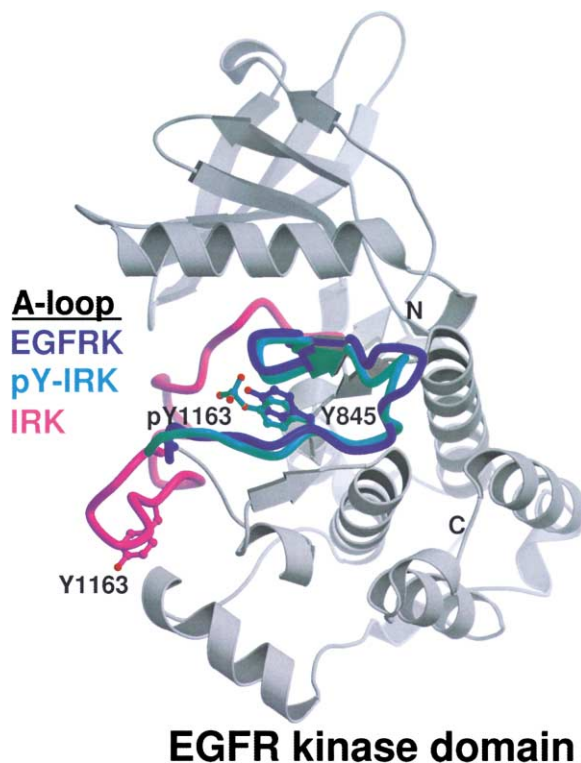pY1163   Y845

Y1163

EGFR kinase domain

Figure 4. The Activation Loop of the EGFR Kinase Is Fixed in an Activated Conformation Even without Phosphorylation

A ribbons representation of the EGFR tyrosine kinase domain is shown (pdb code 1m14), with its activation loop colored blue (Stamos et al., 2002). Superimposed on this view are the configurations of the activation loop in the unphosphorylated insulin receptor (IR) kinase (1irk) in magenta (Hubbard et al., 1994), and the phosphorylated (activated) IR kinase domain (1ir3) in cyan (Hubbard, 1997). The activation loop in the unphosphorylated EGFR kinase domain overlays remarkably well with its counterpart in the activated IR kinase. Y845 of the EGFR kinase domain, and the phosphorylated tyrosine pY1163 of IR kinase, which occupy very similar positions (see text), are marked.

of data support the idea that intracellular domains within a ligand-induced receptor dimer must associate with one another in a particular way for normal receptor activation (Chantry, 1995; Walker et al., 1998), possibly reflecting a requirement for such allosterically induced activation loop changes. This may be the process disrupted by mutations in the intracellular domain "LVI" sequence (residues L955-V956-I957) implicated in ErbB2 activation by homodimerization and by heterodimerization with ErbB3 (Penuel et al., 2002; Schaefer et al., 1999). Another possibility that we cannot exclude is that ErbB receptor kinase activation involves the displacement of inhibitory interactions that were not visualized in this crystallographic study (Stamos et al., 2002), which focused on only the kinase domain and did not include the juxtamembrane or C-terminal regulatory regions (>200 amino acids) of EGFR (Figure 1). If the EGFR kinase domain is truly constitutively active, however, the key event regulated by ligand-induced receptor dimerization must be delivery of substrate to the kinase domain active site. This could involve delivery of a dimerization partner's C-terminal regulatory region for

trans-autophosphorylation, or it could involve the removal of barriers (such as in a preformed inactive receptor dimer) that restrict accessibility of the active site.

Structure of the ErbB2 Extracellular Region Explains Several of Its Unique Properties

The second member of the ErbB family to be identified, ErbB2/HER2, has several unique properties. First, ErbB2 has no known direct ligand, suggesting that it may function primarily as a coreceptor (or heterodimerization partner) for other ErbB receptors that do have ligands (Citri et al., 2003; Yarden and Sliwkowski, 2001). Second, unlike other ErbB receptors, ErbB2 overexpression can cause cell transformation even in the absence of added ligand (Di Fiore et al., 1987; Yarden and Sliwkowski, 2001). The rodent ortholog of ErbB2, named Neu, was recognized over 20 years ago as a potent transforming oncogene product (with a transmembrane domain mutation) in rats treated with ethylnitrosourea (Padhy et al., 1982; Schechter et al., 1984). The unaltered, wild-type, human ErbB2 gene is amplified or overexpressed in a subset of breast cancers (Slamon et al., 1987, 1989), and this correlates with an aggressive tumor phenotype including tumor size, lymph node involvement, high percentage of S-phase cells, aneuploidy, and lack of steroid hormone receptors (Paik and Liu, 2000; Ross and Fletcher, 1998). ErbB2/HER2 amplification or overexpression is also observed in some ovarian, gastric, and salivary cancers (Koeppen et al., 2001; Press et al., 1994). Consistent with its ability to transform cells when overexpressed, these observations suggest that ErbB2 has a high level of constitutive (ligand-independent) activity, and that its expression above a certain threshold level can drive tumor growth (Yarden and Sliwkowski, 2001). These properties of ErbB2 have made it a key target of breast cancer therapies, such as the humanized anti-ErbB2/HER2 Herceptin antibody (Carter et al., 2000).

Three independent X-ray crystal structures of intact (Cho et al., 2003) or truncated (Garrett et al., 2003) sErbB2 have provided satisfying explanations for some of ErbB2's unique properties. The structures of the individual domains are very similar to their sEGFR and sErbB3 counterparts, with the exception of differences in the relative orientations of domain II disulfide-bonded modules (Garrett et al., 2003). Unliganded sErbB2 is unique, however, in the arrangement of its four constituent domains (Figure 5). Even without a bound ligand (there is no known ErbB2 ligand), the sErbB2 structure resembles the extended configuration of sEGFR, suggesting that it may be "autoactivated." There is no intramolecular domain II/IV tether in sErbB2: three of the seven conserved residues important for stabilizing the tether in unactivated sErbB3 and sEGFR are different in ErbB2, presumably reducing the strength of this interaction. Instead, the configuration of sErbB2 is dominated by a unique interface between the two ligand binding domains I and III, which contact one another directly in a way that appears to mimic the bridging of these two domains by bound ligand in activated EGFR (Figure 5). The extensive and highly complementary domain I/III interface buries much of the ErbB2 surface that corresponds to the EGFR ligand binding sites, and
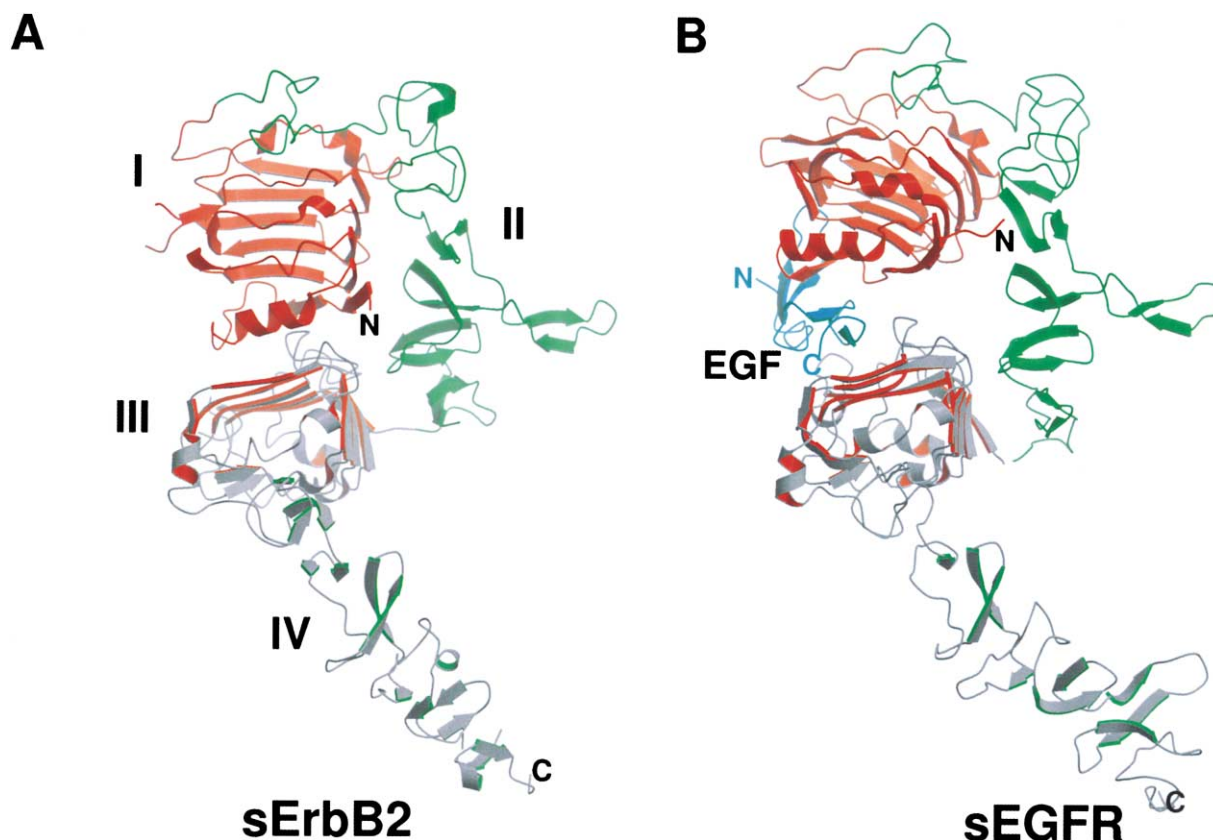
Figure 5. The ErbB2 Extracellular Region Adopts an Extended Configuration that Resembles Ligand-Bound Activated sEGFR

(A) A ribbons representation of human sErbB2 (pdb code 1n8z) is shown (Cho et al., 2003), from which the bound trastuzumab has been omitted. Individual domains are labeled as for other structures (red for domains I and III, green for domains II and IV). The close proximity of domains I and III is notable, as discussed in the text. The sErbB2 dimerization loop is also clearly exposed.

(B) One-half of the sEGFR dimer model from Figure 3 is shown for comparison, referenced to the orientation of domain III in sErbB2.

would undoubtedly block the formation of any receptor•ligand complex that resembles sEGFR•EGF. Furthermore, several residues that play important ligand binding roles in EGFR are replaced in ErbB2 by residues expected to impair ligand binding (e.g., M10, R13, and P15 in ErbB2) (Garrett et al., 2003). These features provide a satisfying explanation for why no ErbB2 ligand has ever been identified: the ErbB2 ligand binding site is both obstructed and mutated.

The finding that ErbB2 constitutively adopts an extended configuration, with its dimerization arm exposed, suggests that ErbB2 is always poised to homodimerize or to form heterodimers with ligand-activated forms of other ErbB receptors. At first consideration, this might explain the unique ability of ErbB2 to transform cells (and to cause cancer) when overexpressed. However, biophysical studies have failed to detect significant sErbB2 dimerization in solution (Ferguson et al., 2000; Horan et al., 1995) or in crystals (where sErbB2 concentrations are $\geq$ 10 mM) (Cho et al., 2003; Garrett et al., 2003). Some constitutive homodimerization of intact ErbB2 may occur in vivo, but is likely to require cooperation of extracellular interactions with additional dimerization sites that have been identified in the ErbB2 transmembrane (Mendrola et al., 2002) and kinase (Penuel et al., 2002) domains. Indeed, the fact that ErbB2 does not

constitutively display maximal activity when expressed in cells, and that it must be overexpressed to quite high levels to cause cell transformation, suggests that ErbB2 homodimerization is rather weak. Accordingly, homodimerization of sErbB2 alone may simply not be strong enough to detect in solution. Indeed, electrostatic calculations suggest that both the dimerization arm of sErbB2 and its docking site in domain II are quite negatively charged and may repel one another (Garrett et al., 2003). The ErbB2 extracellular domain may instead be uniquely suited to its role as the preferred heterodimerization partner (or coreceptor) for other ErbB receptors (Graus-Porta et al., 1997; Karunagaran et al., 1996)—only being able to form (dimerization arm-mediated) heterodimers with other family members that have been activated by their appropriate ligands.

Hetero- versus Homodimerization of ErbB2
To achieve maximum ErbB2 autophosphorylation at normal expression levels, other ErbB receptors in the same cell must be activated (Yarden, 2001). This argues that ErbB2 heterooligomerizes with other ErbB receptors more efficiently than it can homodimerize. Moreover, while sErbB2 homodimerization has never been observed in biophysical studies, there is evidence for NRG-induced heterodimerization (albeit weak) of sErbB2
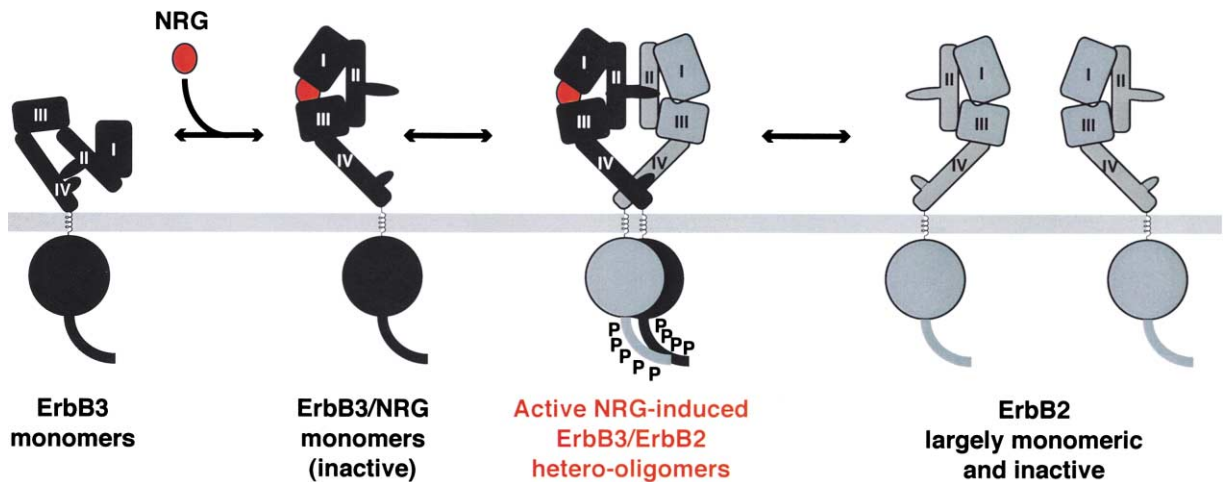
Figure 6. Model for NRG-Induced Heterodimerization of ErbB2 and ErbB3

At left, a tethered ErbB3 monomer is depicted. Binding of NRG (red) is proposed to promote the extended configuration of ErbB3, with the dimerization arm exposed. Extended ErbB3 is thought to form homodimers only very inefficiently. On the right are shown ErbB2 molecules, constitutively in the extended configuration, that are thought to be primarily monomeric (although homodimerization can presumably be driven by substantial overexpression). When NRG-bound ErbB3 molecules are present in the cell membrane, ErbB2 preferentially forms heterooligomers with ErbB3, leading to receptor activation and mitogenic signaling (Citri et al., 2003). Additional interactions involving the transmembrane and kinase domains may also contribute to receptor oligomerization.

with sErbB3 or sErbB4 (Ferguson et al., 2000). A similar preference for heterodimerization is also seen for ErbB3. NRG does not induce sErbB3 homodimerization (Cho and Leahy, 2002; Ferguson et al., 2000; Horan et al., 1995; Landgraf and Eisenberg, 2000), yet may promote sErbB2/sErbB3 heteromer formation (Ferguson et al., 2000), and certainly promotes formation of ErbB2/ErbB3 heteromers in cells (Agus et al., 2002; Citri et al., 2003). ErbB2/ErbB3 heteromers are considered to be the most prevalent and mitogenically potent ErbB receptor/ligand complexes (Citri et al., 2003).

As seen with sEGFR (Elleman et al., 2001), exposure of the dimerization arm is not sufficient for homodimerization of sErbB2 or sErbB3. For sEGFR, the correct register of several domain II dimerization sites (marked in Figure 3) is thought to provide a self-complementary interface that can drive efficient homodimerization. It seems reasonable to suggest that the equivalent surfaces of sErbB2 and extended sErbB3 lack shape (and/or electrostatic) self-complementarity, and that this explains their failure to homodimerize. If ErbB2 and ErbB3 instead present surfaces that pack well against (or complement) one another, this could explain their preference for heterodimerization.

A simple hypothesis for ligand-induced ErbB receptor heterodimerization is depicted schematically in Figure 6 (see also Citri et al., 2003). In an unactivated cell, ErbB2 molecules are extended but predominantly monomeric. ErbB3 molecules are mostly tethered and monomeric. When NRG is added, it binds to ErbB3 and promotes adoption of the extended configuration. Rather than forming homodimers, the extended ErbB3 molecules preferentially heterodimerize with ErbB2, and a potent mitogenic response is induced (Citri et al., 2003). According to this hypothesis, the extended forms of EGFR and ErbB4, which both homodimerize efficiently and can also form heterodimers with ErbB2, should possess

domain II interaction surfaces that are capable of either interacting with themselves or with ErbB2. In these cases, mixtures of homodimers and heterodimers should form. Confirmation (or otherwise) of this view awaits determination of the structure of a heteromeric sErbB receptor complex.

Structural Basis for Action of Therapeutic Antibodies Directed to ErbB Receptor Extracellular Regions

The structural view outlined here suggests several ways to inhibit or prevent ErbB receptor activation—an important aim in current cancer research (Arteaga, 2003). So far, all of the promising therapeutic approaches that target ErbB receptor extracellular regions are antibody based (Arteaga, 2003; Sliwkowski et al., 1999). Humanized or chimeric antibodies raised against the extracellular regions of EGFR and of ErbB2 have shown clinical promise and/or value (Arteaga, 2003; Ranson and Sliwkowski, 2002; Sliwkowski et al., 1999). Several of the anti-EGFR antibodies are in clinical trials. Some of these inhibit EGF binding and act as antagonists, but their precise epitopes and modes of action are not yet clear. The best-known therapeutic anti-ErbB receptor antibody is trastuzumab (Herceptin), which was developed as a therapeutic option for women whose tumors overexpress ErbB2 (Carter et al., 2000; Slamon et al., 2001) and approved by the FDA in 1998. The efficacy of trastuzumab requires the presence of an intact Fc region, implying a role for antibody-dependent cellular cytotoxicity (ADCC) in its action. Trastuzumab binds strongly to tumor cells that overexpress ErbB2. Since it contains a human IgG_1 Fc region, the antibody will then serve as a "beacon" for infiltrating immune effector cells, leading to ADCC. Indeed, trastuzumab has been shown to have very little antitumor activity in mice that lack Fcγ receptors (Clynes et al., 2000).

The structure of the trastuzumab Fab fragment bound

to sErbB2 revealed that its epitope is toward the C terminus of domain IV (Cho et al., 2003). The trastuzumab binding site includes the counterparts of the domain IV residues that participate in the intramolecular tether observed in sEGFR and sErbB3. Trastuzumab actually acts as a weak agonist of ErbB2 in cell culture experiments (Sliwkowski et al., 1999), and does not significantly impair the ability of ErbB2 to form heterodimers with ErbB3 (Agus et al., 2002). It therefore seems reasonable to argue that trastuzumab does not block important sites of receptor-receptor interaction. Domain IV does include, however, the cleavage site used when the ErbB2 extracellular region is released from the membrane by metalloproteinases. Once the extracellular region is removed, the remnant ErbB2 transmembrane and intracellular domains are thought to self-associate (and to interact with uncleaved ErbB2), resulting in constitutive kinase activation (Molina et al., 2001). Intriguingly, trastuzumab blocks this proteolytic cleavage event, and this is thought to be an important component of its antitumor activity. The resulting reduction of ErbB2 signaling, together with the Fc-Fcγ interaction outlined above, are likely to explain trastuzumab's action.

The sErbB structures clearly suggest epitopes that should be targeted by future therapeutic antibodies. The most obvious is the domain II dimerization arm itself. Other parts of domain II may be just as fruitfully targeted. The anti-ErbB2 antibody called pertuzumab (Omnitarg or 2C4) has been shown to disrupt ErbB2/ErbB3 interactions efficiently—both as a monovalent Fab fragment and as the intact antibody (Agus et al., 2002). A recent crystal structure of the complex between sErbB2 and the pertuzumab Fab fragment (Franklin et al., unpublished data) provides an explanation for this. Pertuzumab binds to domain II of sErbB2, primarily through contacts between the V_H domain of the antibody and residues just C-terminal to the sErbB2 dimerization arm (as well as two residues at the tip of this arm). The counterparts of many of the sErbB2 residues contacted by pertuzumab are involved in sEGFR dimerization. The ability of pertuzumab to inhibit ErbB2/ErbB3 heterodimerization is therefore likely to result from its occlusion of residues directly involved in interreceptor interactions, plus (perhaps more importantly) a steric blockade to heterodimerization. It is to be expected, following the development of the structural understanding outlined here, that other similarly effective (and targeted) therapeutic anti-ErbB antibodies will be forthcoming.

Conclusions

The recent structural studies of ErbB receptor extracellular regions have provided new insights into ligand binding specificity, as well as receptor latency, affinity, and modes of oligomerization. It is at last possible to envisage mechanisms by which ligand binding induces dramatic domain rearrangements that promote receptor homo- and/or heterodimerization, and consequent activation of the intracellular kinase domain. While the current data have enormously advanced our understanding of EGFR family monomers and dimers, there is still much to be learned about the role of conformational reorientation and oligomerization in kinase activation. Hints from the crystallographic studies of the possible nature of "preformed" ErbB receptor dimers, not discussed here, also need to be followed up. The structural information suggests explanations for the specific biological roles of each ErbB receptor; in particular the coreceptor role for ErbB2. The visualization of quite different configurations for the monomeric and dimeric extracellular regions has also revealed sites for targeting the binding of agents that will inhibit receptor activation, and thus provides exciting opportunities for the design of novel anticancer agents.

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