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Interaction of antibodies with ErbB receptor extracellular regions

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Abstract

Antibodies to the extracellular region of the ErbB receptors have played key roles in the development of a mechanistic understanding of this family of receptor tyrosine kinases. An extensively studied class of such antibodies inhibits activation of ErbB receptors, and these antibodies have been the focus of intense development as anti-cancer agents. In this review we consider the properties of ErbB receptors antibodies in light of the current structure-based model for ErbB receptor homo- and hetero-dimerization and activation. Crystal structures of the Fab fragments from five different inhibitory antibodies in complex with the extracellular regions of EGFR and ErbB2 have been determined. These structures highlight several different modes of binding and mechanisms of receptor inhibition. Information about antibody interactions with the structurally well-characterized soluble extracellular regions of ErbB receptors can be combined with the rich knowledge of the effects of these antibodies in cultured cells, and *in vivo*, to provide insights into the conformation and activation of ErbB receptors at the cell surface.

Keywords

EGFR/ErbB1; ErbB2/HER2; Antibody; Trastuzumab/Herceptin; Cetuximab/Erbitux; ErbB receptor inhibition

Introduction

Antibodies have played a crucial role in understanding ErbB receptors since the early mechanistic studies of this family of receptor tyrosine kinases (RTKs). Antibodies were essential for the generation of purified EGFR that was used to demonstrate that ligand-induced dimerization is a critical first step in receptor activation [1]. Others antibodies have provided clues as to the nature of the two ligand affinity “classes” of receptors that exist at the cell surface [2,3]. For ErbB2 (also known HER2/neu), antibodies have also played a key role in establishing the identity and role of this receptor [4,5]. ErbB2 antibodies were found to reverse the phenotype of transformed cells by binding to and down-modulating this oncogenic protein [6]. This confirmed the link between the neu oncogene and malignancy, and provided proof of concept that antibodies could have antitumor activity [7].

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In the early 1980s, a number of groups generated monoclonal antibodies to the extracellular region of EGFR using as immunogen A431 epidermoid carcinoma cells, which express high levels of cell surface epidermal growth factor receptor (EGFR). The resulting antibodies display various properties. Some had no effect on growth factor activation [8], while others induced receptor aggregation and mimicked the effects of ligand stimulation [9,10]. A third class of antibodies blocked the ability of growth factor to activate the receptor [11,12]. Antibodies of this third class have received substantial attention as potential inhibitors of EGFR activation in human tumors [11,12]. It has been clear for many years that there is a correlation between aberrant activation of members of the ErbB receptor family and the development and progression of cancers [13,14]. Both the extracellular and intracellular regions of EGFR and ErbB2 are targets of therapeutic agents in active clinical use and/or development [15,16]. In 2003, when the first special issue on the ErbB/EGF system was published in *Experimental Cell Research*, the ErbB2 targeted antibody drug trastuzumab/Herceptin had been approved for use in ErbB2 positive breast cancers, and several anti-EGFR directed antibody drugs were in clinical trials. There are now three EGFR antibodies approved for use in various clinical settings (Table 1), and numerous other antibodies against this family of receptors are the focus of active clinical trials. Many excellent reviews focus on the development and clinical application of monoclonal antibodies against the extracellular regions of ErbB receptors [7,15–18]. In this review we consider the interactions of antibodies with the extracellular region of ErbB receptors in light of structure-based models of ErbB receptor homo- and hetero-dimerization.

Structural studies in the past 6 years have led to a model for ligand-induced homo- and hetero-dimerization and activation of ErbB receptors, the details of which have been extensively reviewed [19–21]. Recent developments in understanding mechanisms of ErbB receptor activation are discussed in an accompanying review in this issue [22]. The salient points of this model for the discussion of antibody mediated inhibition of ErbB receptors are summarized in Figure 1. For three of the four members of the ErbB receptor family, EGFR/ErbB1, ErbB3/HER3 and ErbB4/HER4, the unliganded extracellular region adopts a “tethered” conformation in which domains II and IV interact [23–25]. The ligand bound form of the extracellular region has only been crystallographically observed for EGFR [26,27] and, in this state, the extracellular region of the receptor adopts a very different conformation. Ligand binds between domains I and III of the receptor molecule, and drives exposure and remodeling of a dimerization interface on domain II. The fourth member of this family of receptors, ErbB2, has no known soluble ligand and is a structural outlier among this family of four receptors. The unliganded conformation of ErbB2 does not adopt the tethered conformation. Rather, ErbB2 adopts an extended arrangement of domains similar that of the ligand bound form of EGFR [28,29]. Based on these structures a generalized mechanism of ligand induced homo- and hetero-dimerization has been proposed [19] and is shown schematically in Figure 1. This mechanism of activation of ErbB receptors has substantial implication for possible modes of binding by antibodies that could inhibit or modulate receptor activation.

Antibodies against the extracellular region of ErbB receptors

In the following sections we briefly review a selection of the antibodies against the extracellular regions of ErbB receptors that are important inhibitors of receptor function, or that have provided opportunities to probe receptor activation mechanisms. Properties of key antibodies are also summarized in Table 1.

EGFR antibodies

mAb 225 (chimerized to IMC-C225/cetuximab/Erbitux)—Monoclonal antibody 225 was one of several antibodies raised by inoculation of mice with A431 epidermoid carcinoma cells by a group led by Prof. John Mendelsohn (University of Texas M.D. Anderson Cancer Center). Three antibodies (mAbs 225, 528 and 579) were characterized that inhibited EGF

binding to the receptor, blocked ligand-dependent receptor activation [11,12,30] and inhibited cellular proliferation *in vitro* and *in vivo* [31,32]. Monoclonal antibody 225 was selected to generate a human/mouse chimeric molecule for clinical development [33]. The resulting chimeric antibody, IMC-C225/cetuximab, originally developed by ImClone Inc., was first approved for therapeutic application in 2004. It is marketed under the trade name Erbitux by Bristol Myers in the US and Merck KGaA elsewhere. Cetuximab is being investigated in multiple clinical trials to broaden its clinical uses. Clinical studies with this antibody have been extensively reviewed (see for example [34–36] and references therein).

mAb 425 (humanized to EMD 72000/matuzumab)—Independently, a group at the Wistar Institute (Philadelphia) also generated a mouse monoclonal antibody against the extracellular region of EGFR using A431 cells. Like cetuximab, mAb 425 blocks binding of EGF and TGFα to A431 cells, blocks EGFR activation [37] and inhibits tumor growth in mouse models [38]. A humanized version of mAb 425, matuzumab/EMD 72000 (Merck KGaA) has progressed to Phase II clinical trials to treat a range of cancers, both alone and in combination therapy [39,40].

IMC-11F8—This fully human antibody was constructed using an isolate from a non-immunized human Fab phage display library [41,42]. The Fab from this library was selected for high affinity binding to the EGFR on A431 cells, and for its ability to compete with cetuximab for binding to these cells [42]. IMC-11F8 inhibits EGFR activation in several cell-lines [42,43], blocks tumor growth in xenograft models [44,45], and has performed well in phase I clinical trials [46]. Now in Phase II clinical trials, IMC-11F8 holds promise as a next generation cetuximab.

ABX-EGF/Panitumumab/Vectibix and HuMax-EGFr/Zalutumumab—These two antibodies have been developed more recently from transgenic mice that express fully human antibodies [47]. ABX-EGF binds to EGFR with higher affinity than cetuximab, blocks ligand binding and receptor activation, and has potent anti-tumor activity in model systems [48]. It is the focus of multiple ongoing clinical trials and has been approved for use in colorectal cancer ([49] and references therein). Initially developed by Abgenix, ABX-EGF is now being developed and marketed by Amgen under the trade name Vectibix. ABX-EGF is an antibody of subtype IgG2 and does not stimulate robust antibody dependent cellular cytotoxicity (ADCC), an immune effector mechanism that contributes to the antitumor activity of many antibodies [50,51]. HuMax-EGFr (originally named mAb 2F8) was developed by GenMab using a different transgenic mouse platform (generating IgG1 antibodies), and using both A431 cells and purified receptor as immunogen [52]. The preclinical characteristics of this antibody are similar to others that have shown clinical promise, with excellent anti-tumor activity at low dose. Zalutumumab is in accelerated clinical trials in a number of settings [53].

mAb 108 and mAb 2e9—These two mouse monoclonal antibodies have not been developed for clinical application. Rather these have provided interesting clues about the binding of ligand to cell surface EGFR. Monoclonal antibody 108 was raised using CHO cells that overexpress a human EGFR truncation variant lacking the intracellular domain [2]. These cells were used as an alternative to A431 cells as they lack certain highly antigenic carbohydrate groups. Mouse monoclonal antibody 108 selectively blocks binding of EGF to the high-affinity sub-population (5–10%) of EGF binding sites (with K_D < 100 pM) observed on the surface of EGFR-expressing cultured cells, without affecting binding to the majority (90 – 95%) of lower affinity EGF binding sites (K_D of 2–12 nM) [2]. Like cetuximab, mAb 108 is effective in inhibiting growth of human tumors in mouse xenografts, demonstrating that this antibody blocks proliferative EGFR signaling *in vivo* [54]. *In vitro*, early cellular responses to physiologically relevant concentrations of EGF, such as receptor phosphorylation and alterations in

intracellular Ca^{2+} levels, are blocked by mAb 108 in a manner that coincides with the loss of higher affinity EGF-binding sites. Monoclonal antibody 2E9 was generated using A431 cell membranes as an immunogen [55]. By contrast with mAb 108, mAb 2E9 binding to cell surface EGFR blocks only the *low* affinity EGF binding sites, without influencing the high affinity sites [3,55], and does not block cell proliferation. It should be noted that both mAbs 108 and 2E9 bind to all the receptors at the cell surface – they do not selectively bind to *only* "high" or "low" affinity receptors. Rather the binding of these antibodies to all receptors is able to modulate cell surface ligand binding.

mAb 806—This mouse monoclonal antibody was generated using cells expressing EGFRvIII, also known as de2-7 EGFR, as antigen [56]. EGFRvIII is the most common gene disruption in the region containing the exons for the extracellular region of EGFR, and is found in about 25 % of glioblastomas, as well as in a number of solid tumors [57]. Exons 2–7 are deleted resulting in the EGFRvIII protein that (i) lacks amino acids 6–273 of the mature protein, (ii) has a glycine following amino acid 5, and (iii) has an unpaired cysteine at amino acid 16 (amino acid 283 of mature EGFR). In addition to binding to cell surface EGFRvIII, mAb 806 also binds to a fraction (< 10%) of wild-type EGFR in cells expressing elevated levels of the receptor, but not to the wild-type EGFR in normal tissue or to tumor cells that express low levels of EGFR [58]. Consistent with this binding profile, this antibody shows antitumor activity against xenografts expressing EGFRvIII or high levels of EGFR. Treatment of cultured glioblastoma cell lines with mAb 806 leads to a decrease in EGFRvIII phosphorylation and reduced cellular proliferation [56]. A chimeric version of 806 (c806) has been engineered and has performed well in phase I trials [59]. Monoclonal antibody c806 appears to concentrate in the tumors of several patients in this trial, consistent with the argument that this antibody shows increase binding to tumors compared with normal tissue.

ErbB2 antibodies

4D5 (humanized to trastuzumab/Herceptin)—In the late 1980s the case for developing an antibody-based anti-ErbB2 therapeutic was strong. It had recently been identified that ErbB2 is overexpressed in almost 30 % of breast tumors, and that this correlates with aggressive disease progression [60]. Antibodies raised to mouse cells transformed with rat ErbB2 had been shown to reverse the transforming effects of the neu oncogene [4], and EGFR targeted antibodies with anti-tumor activity had been identified [32]. Researchers at Genentech generated a panel of antibodies against human ErbB2 [61]. These antibodies were tested against a range of cell lines and in xenograft studies. One antibody, mAb 4D5, was selected as the top candidate with therapeutic potential against ErbB2 overexpressing tumors ([18] and references therein). A human/mouse chimeric version of 4D5 was generated and, following rapid successful clinical trials (from Phase I to approval in 6 years), was FDA-approved for use against ErbB2 positive breast cancers in 1998. An excellent review detailing the progress of trastuzumab from concept to clinic has recently been published [18].

2C4 (humanized to pertuzumab/Omnitarg)—One of the antibodies from the panel generated by Genentech was found to have quite different properties form those of mAb 4D5 and to have a non-overlapping epitope [61,62]. This antibody blocks ligand-dependent ErbB2 activation and has been the focus of particular attention for its therapeutic potential in breast and prostate cancers that do not express high levels of ErbB2. Monoclonal antibody 2C4 is a potent inhibitor of neuregulin-induced receptor phosphorylation and downstream signaling in cell lines with low levels of ErbB2, and has antitumor activity in xenograft models with this same receptor expression profile [62]. A humanized version of this antibody is in Phase II clinical trials [36].

Mechanisms of inhibition of ErbB receptor activation by therapeutic antibodies

The mechanism of activation of ErbB receptors, illustrated in Figure 1, suggests several modes of binding by an antibody that could inhibit ligand-dependent activation of these receptors. These mechanisms can be conceptually broken down: (i) stabilization of the tethered conformation, (ii) block of the domain rearrangement required to attain the extended state, (iii) direct block of ligand binding (iv) direct block of receptor dimerization. Co-crystal structures have been determined of the Fab fragments from five different clinically relevant antibodies in complex with the extracellular regions of EGFR and of ErbB2 (Figure 2) [28,63–66]. As summarized in Figure 3 and discussed below, these structures illustrate aspects of these different mechanisms of ErbB receptor inhibition.

Direct competition for ligand binding

The X-ray crystal structure of the Fab fragment from cetuximab in complex with sEGFR shows that the epitope for this antibody lies on domain III of EGFR and overlaps substantially with the EGF binding site on that domain [65] (Figure 2A & 4A). Direct occlusion of the ligand-binding site is the primary mechanism of inhibition by this antibody (Figure 3A). The epitope for the fully human inhibitory antibody mAb IMC-11F8 has also been crystallographically defined and is almost identical to that of cetuximab, although the sequences of the complementarity determining regions (CDRs) for these two antibodies are quite different [64].

Based on these co-crystal structures, and on the structures of sEGFR in complex with EGF [27] and TGFα [26], alterations were made in the domain III binding site of EGFR that disrupt ligand binding, cetuximab/IMC-11F8 binding, or both [64,65]. This information was used to glean information about the epitopes of several other antibodies that are performing well as EGFR targeted drugs [67,68] (Figure 4B), and these epitope mapping data have been corroborated with cross-competition between different antibodies for binding to cell surface EGFR [69]. The epitopes for panitumumab and zalutumumab both overlap substantially with that of cetuximab, and with the EGF binding site on domain III. It is highly likely that the mechanism of inhibition of ligand dependent EGFR activation by these two antibodies is similar to that of cetuximab, although the precise orientation of these two Fab fragments on sEGFR has not been crystallographically defined.

Steric block of the conformation change required for high affinity ligand binding and dimerization

As discussed above the primary mechanism for cetuximab-mediated inhibition of ligand induced EGFR activation is the direct block of ligand binding. However the structure suggests an additional mechanism that may also play a role in the antitumor activity of cetuximab. The orientation of the Fab when bound to its epitope on domain III sterically blocks the receptor from adopting the extended conformation required for dimerization [65]. We speculated that this represents a meaningful component of the inhibitory mechanism in tumors that express aberrantly high levels of EGFR, where ligand-independent dimerization and activation of the receptor could contribute to tumorigenesis [16].

Structural studies of the Fab fragment from another clinically significant antibody, matuzumab, show that steric block of receptor conformational change can be the sole mechanism of inhibition of ligand induced EGFR dimerization and activation [66] (Figure 2B). The epitope for matuzumab also lies on domain III of EGFR, but matuzumab binding does not occlude the ligand-binding site on the receptor. In this sense, matuzumab is quite different from cetuximab, IMC-11F8, panitumumab and zalutumumab (Figure 4A & B). When bound to EGFR, the

matuzumab Fab is oriented such that it sterically prevents domain II from adopting the conformation required for high affinity ligand-binding and receptor dimerization [66]. Notably, matuzumab binding prevents interactions between domains II and III that are known to be critical for receptor activation [27,70]. It is highly likely that this "conformational-restriction" mechanism will be exploited by antibodies that bind to other epitopes either on domain III or elsewhere on the receptor.

Stabilization of the inactive tethered conformation

From the structure-based mechanism presented in Figure 1, it is clear that an agent that locks the receptor in the tethered conformation would be an effective inhibitor. The identification of such a mode of binding based on studies of the soluble extracellular region of the receptor has not been possible since no direct assay for the stability of the tethered conformation has been established. Disrupting all structurally defined interactions that characterize the tethered conformation does not perturb the solution conformation of the protein [71]. The electron microscopy based technique of Protein Tomography has been used to study the conformation of cell surface EGFR [68]. These studies show evidence of different conformations of unliganded cell surface EGFR, including some that are consistent with the crystallographically defined tethered state, and others that appear to be more extended [68]. Significantly, in the presence of zalutumumab only the more compact, tethered conformation of EGFR is observed. These data suggest that binding of zalutumumab to domain III may stabilize the tethered conformation, although the precise molecular basis for this stabilization is not yet clear.

Direct block of receptor dimerization

It is clear that an agent that directly blocks formation of the receptor mediated contacts in the activated dimer would be an effective inhibitor. Precisely this mechanism is suggested by the structure of pertuzumab in complex with the extracellular region of ErbB2 [63]. Pertuzumab binds to an epitope on the domain II dimerization arm of ErbB2 (Figure 3C), effectively blocking the ability of ErbB2 to form heterodimers with other ErbB receptors (Figure 2C). The identification of the epitope for pertuzumab on the dimerization arm of ErbB2 is convincing corroboration of the model for heterodimerization presented in Figure 1C. The soluble extracellular regions of ErbB receptors do not form stable heterodimers [72] and the structure of an ErbB receptor heterodimer has yet to be determined. Pertuzumab blocks formation of cell surface heterodimers of ErbB2 with both ErbB3 and with EGFR [62,73], implying that the domain II dimerization arm of ErbB2 is required for formation of these heterodimers. By contrast, pertuzumab does not inhibit the ligand-independent activation of overexpressed ErbB2 [62], suggesting that in this context the activation of the kinase domain of ErbB2 is not triggered by formation of a domain II mediated homodimer akin to the homodimer observed structurally for ligand-bound EGFR.

The binding of an inhibitory agent to the domain II dimerization arm of EGFR has not yet been described, however such an agent would clearly be a potent inhibitor of ligand induced homodimerization of EGFR. It is possible that the lack of such an inhibitor may result from the relatively limited selection procedures that have been used to generate the current repertoire of inhibitory antibodies. A431 cells have been the primary immunogen or selection agent used for almost all antibodies to the extracellular region of EGFR. This may bias the array of conformations of the receptor presented for antibody selection.

Insights from the binding of trastuzumab to ErbB2

Trastuzumab prevents proliferation of cells that overexpress ErbB2 [18]. In these cells ErbB2 is constitutively active, and there is no requirement for an activating ErbB ligand. The antitumor activity of trastuzumab is multifaceted in animals, with critical roles arising from receptor downregulation and ADCC (antibody dependent cellular cytotoxicity) [18,36]. Here we

consider the insights that come from the structural definition of the trastuzumab-epitope on domain IV of ErbB2 [28].

Domain IV has been suggested to play a direct role in the stabilization of the ligand-induced dimers of EGFR [19,25], although the energetic contribution of this interaction is not large [70]. If domain IV of ErbB2 plays a role in stabilizing heterodimers involving ErbB2, then it might be anticipated that trastuzumab would block their formation. In cells expressing modest levels of ErbB2, trastuzumab does not block the formation of neuregulin-induced heterodimers with ErbB3 [62,73], but the formation of ErbB2/EGFR heterodimers appears to be inhibited [73]. One interpretation of these observations is that the conformations of the heterodimers formed with ErbB3 and EGFR differ such that only ErbB2/EGFR heterodimers are blocked by trastuzumab binding to ErbB2. Domain IV interactions might be more important in one case than the other. This indirect evidence supports the model that heterodimers of ErbB receptors - at least between ErbB2 and EGFR - will indeed resemble the structure of the EGFR homodimer. Confirmation of this model awaits a detailed structural description of an ErbB receptor heterodimer.

As mentioned above, activation of overexpressed ErbB2 is unlikely to occur through formation of domain II mediated ErbB2 homodimers. One mechanism of inhibition of ErbB2 activation that is clearly explained by binding of trastuzumab to domain IV of ErbB2 is prevention of ectodomain shedding [74]. Cells that overexpress ErbB2 exhibit increased ectodomain shedding and this has been linked to oncogenesis [74]. Trastuzumab occludes the proteolytic cleavage site in the juxtamembrane region of the ErbB2 [28] and would thus block activation of the intracellular kinase domains that follows cleavage.

Insights into the activation of ErbB receptors at the cell surface from studies with antibodies

High and low affinity binding sites for growth factor at the cell surface

The binding of EGF to cell surface EGFR produces curvilinear Scatchard plots, the molecular explanation of which remains a matter of debate, and considerable interest. The predominant view is that there are two classes of ligand binding site at the cell surface [75,76] – a low population of high affinity sites (K_D < 100 pM) and a majority lower affinity class of sites ($K_D \approx$ 10 nM) [2], although alternative explanations, such as negative cooperativity in ligand binding, have also been suggested [77,78]. Adding weight to the argument for two populations of receptors are mAb 108 and mAb 2E9. While mAb 108 selectively blocks the high-affinity class of binding sites, mAb 2E9 blocks only the low affinity receptors (leaving the high-affinity sites intact). The nature of the difference between high affinity and low affinity EGF binding sites is not at all clear [79]. However, it appears that the high-affinity class of EGF receptors is the most important in cellular EGFR signaling [2,3]. Several suggestions have been made as to what defines the high- and low-affinity sites, including the possibility that high-affinity sites represent pre-formed receptor dimers, and low-affinity sites represent monomers [79]. Attempts to reconcile the two affinity classes with the structure-based model [19] have led to conflicting conclusions [80–82]. Structural definition of the epitopes and modes of binding to EGFR of mAb 108 and 2E9 would likely shed substantial light not only on how these antibodies achieve these specific inhibitory effects, but also on the origin of these effects on ligand binding to cell surface EGFR.

Conformation of EGFR at the cell surface

It is clear that the preferred solution conformation of the extracellular region of EGFR, ErbB3 and ErbB4 is the tethered configuration, and that of ErbB2 is extended [71]. The conformation of these receptors at the cell surface is less clear. All antibodies discussed in this review bind

to EGFR or to ErbB2 at the cell surface, or at least to a sub-population of these receptors. Can the definition of the epitopes and modes of binding for these antibodies shed light on conformations of ErbB receptors at the cell surface? Binding of the Fab fragments from cetuximab, trastuzumab and pertuzumab to the entire extracellular regions of EGFR and ErbB2 does not perturb the conformation of these receptors. These epitopes must be exposed at the cell surface as they are in the solution and crystal conformations of these receptors. Further, recent Protein Tomography studies of binding of zalutumumab to EGFR at the surface of A431 cells are consistent with its binding to a tethered EGFR [68].

Monoclonal antibody 806 has been suggested to offer a unique insight into the conformational transition from tethered to extended receptor at the cell surface [82,83]. The epitope for this antibody has been mapped by deletion analysis to a short loop between amino acids C287 and C302, and amino acids in this loop that are critical for binding have been defined using yeast phage display [83,84]. This epitope is occluded both in the tethered and extended, dimeric, conformations of EGFR. This explains satisfyingly why this antibody does not react with wild-type EGFR in normal tissue or on cells that express low levels of this receptor. Monoclonal antibody 806 does bind to a fraction (< 10%) of EGFR at the surface of cells expression high levels of this receptor [85] – clearly this fraction of the receptor does not adopt either of the crystallographically defined receptor conformations. It has been suggested that the population of EGFR to which mAb 806 binds represents an intermediate between tethered monomer and extended ligand-bound dimer [82,83], and a model of such a structure has been proposed [86]. Since monoclonal antibody 806 is not conformationally sensitive - it binds to denatured EGFR [85] - this population may instead be misfolded. It remains to be confirmed whether the mAb 806 reactive population of receptors truly represents an intermediate conformation of wild-type EGFR, or simply a population of partially denatured receptors that accumulates on the cell surface under conditions of aberrantly high EGFR expression. Irrespective of the precise nature of the EGFR to which mAb 806 binds, this population of mAb 806 reactive EGFR serves as a tumor marker and lends mAb 806 a distinctive therapeutic potential.

Conclusion

The current repertoire of antibodies to the extracellular region of ErbB receptors has provided valuable information on the function of these receptors in both normal and cancerous cells. The structures of the Fab fragments of five different antibodies in complex with ErbB receptor extracellular regions highlight four distinct modes of binding that can inhibit receptor activation. It is likely that other inhibitory modes of binding are possible, but different strategies may be needed to isolate antibodies with alternate binding properties. ErbB3 and possible ErbB4 may also play a role in development and progression of human cancers [87–89] - only a few antibodies to the extracellular regions of these receptors have been investigated [90, 91]. Combinations of different ErbB targeted antibodies can have synergistic effects [69,92, 93], and the engineering of bispecific antibodies with dual epitope recognition is another promising direction [36,94]. Whether new antibodies against ErbB receptors that have different modes of binding will prove to be of therapeutic value remains to be evaluated. Certainly such antibodies will be exploited to test and extend the current understanding of ErbB receptor activation.

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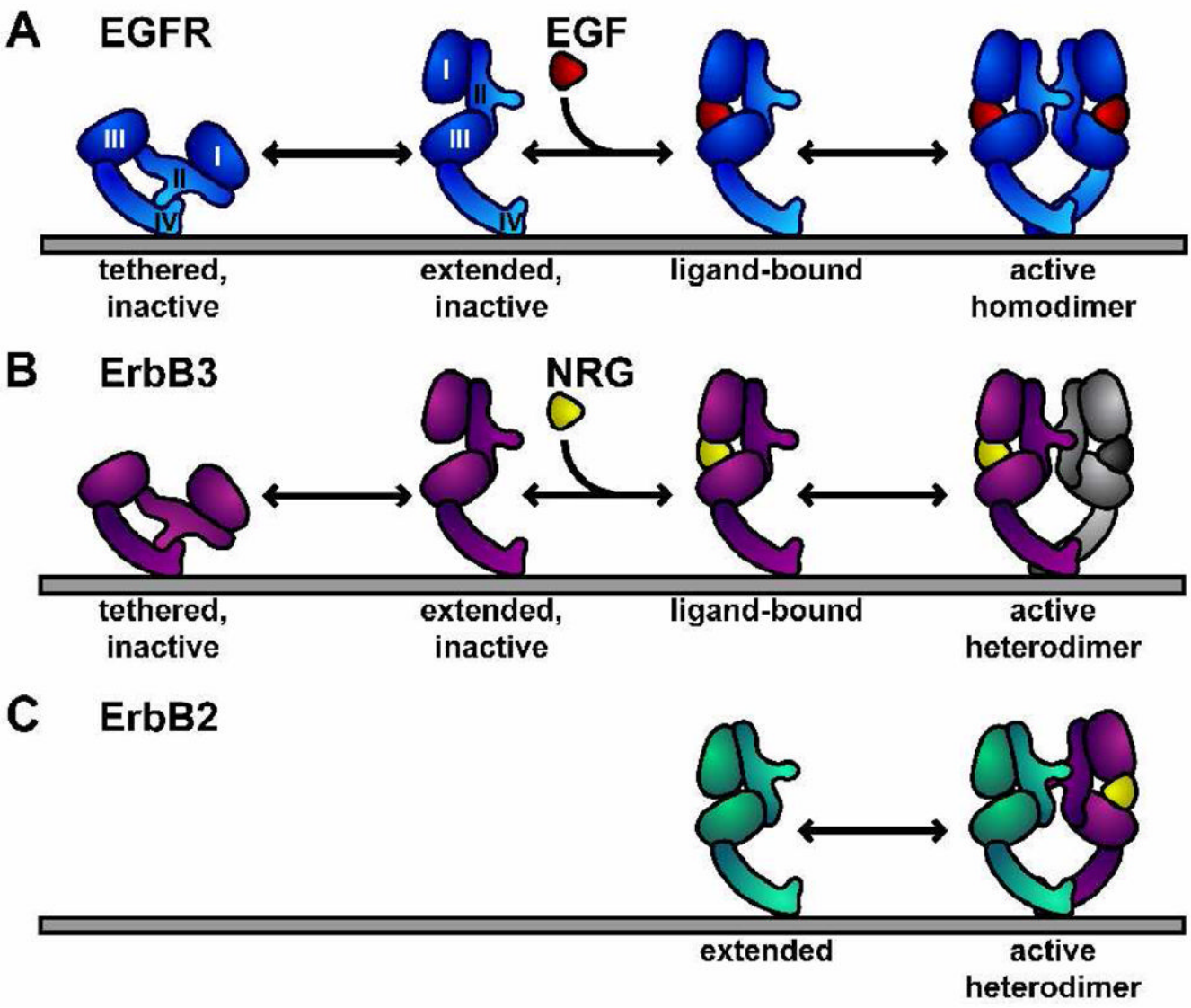

Figure 1. Mechanism of homo- and hetero-dimerization of the extracellular regions of ErbB receptors [19]

A The unliganded state of the EGFR extracellular region (sEGFR) adopts a tethered configuration of domains that is quite distinct from the domain arrangement in the ligand-induced dimer. Conceptual structural intermediates are shown: (i) an extended, unliganded monomer and (ii) an extended ligand-bound monomer. Ligand binding and dimerization are highly cooperative and such intermediates are not significantly populated in solution studies of sEGFR. Crystal structures have been observed of the tethered monomer [25] and ligand-induced dimers comprising domains I, II, III and the first disulfide-bonded module of domain IV [26,27]. The remainder of domain IV is modeled as previously described [25].

B For ErbB3 and ErbB4 structures of tethered monomers have been determined that resemble that of EGFR [23,24]. ErbB3 extracellular region does not homodimerize in solution [71] or in cells [72,95]. Small angle X-ray scattering studies show that neuregulin does promotes the formation of an extended ligand-bound monomer [71]. The model predicts that ErbB4 will form homodimers as in A, and that both ErbB3 and ErbB4 can form heterodimers with at least a subset of other ligand-bound ErbB receptors.

C Unliganded ErbB2 adopts an extended structure with a domain configuration similar to that of extended (ligand-bound) sEGFR [28,29]. ErbB2 is proposed to heterodimerize with other ligand-bound extended ErbB receptors using a similar, largely domain II-mediated, dimerization interface.

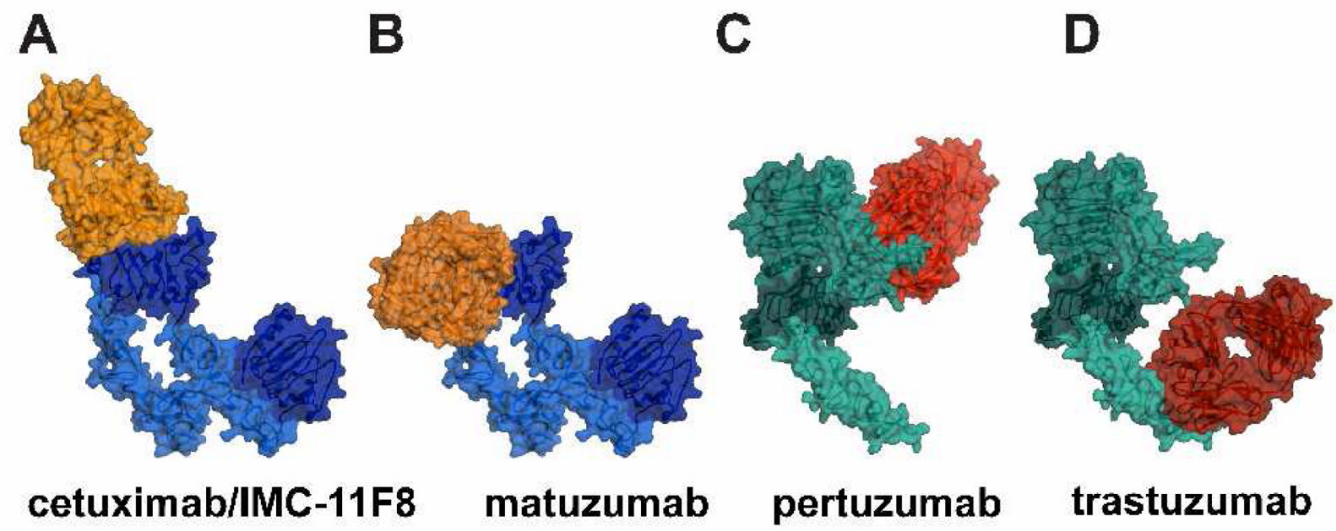

Figure 2. Co-crystal structures of ErbB extracellular regions with Fab fragments from inhibitory antibodies

A molecular surface representations of the Fab fragment from each antibody in complex with the entire extracellular region of EGFR (blue) or ErbB2 (green) are shown.

A. Cetuximab (orange) binds to domain III of EGFR [65] (pdb id. 1yy9).

B. Matuzumab (orange) binds to a different epitope on domain III. The co-crystal structure contained only domain III [66] (pdb id. 3c09), the other domains of EGFR are modeled using pdb id. 1yy9.

C. Pertuzumab (red) binds to the domain II dimerization arm of ErbB2 [63] (pdb id. 1s78).

D. Trastuzumab (red) binds to domain IV of ErbB2 [28] (pdb id. 1n8z).

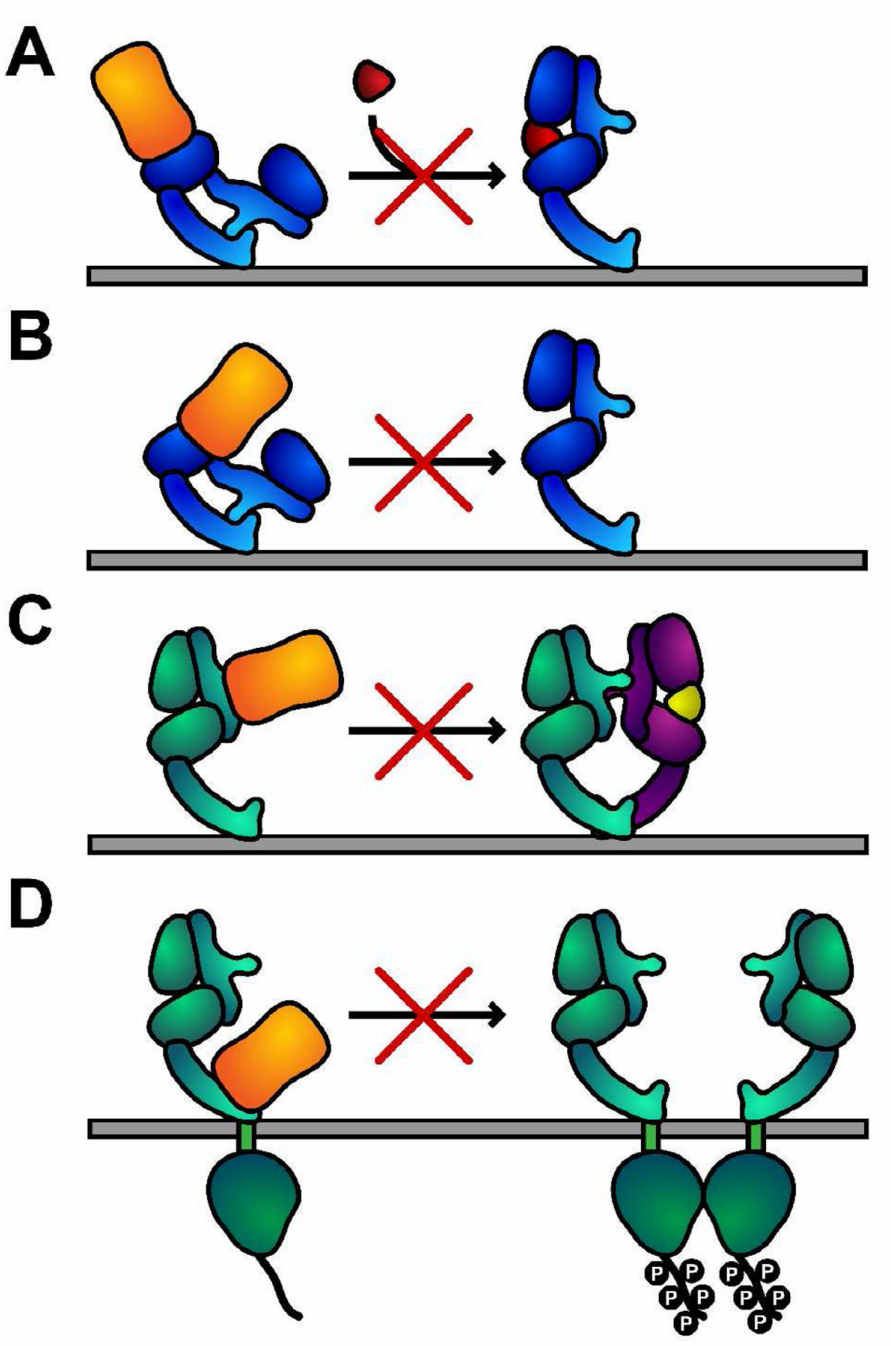

Figure 3. Mechanisms of inhibition of ErbB receptor activation by antibodies that bind to the extracellular region

A. Antibody binding directly occludes the ligand-binding site – this mode of inhibition is important for cetuximab, IMC-11F8, panitumumab and zalutumumab.

B. Antibody sterically prevents the receptor from adopting the conformation required for high-affinity ligand binding and dimerization, without directly occluding a ligand-binding site. This mode is observed for matuzumab.

C. Antibody binding directly prevents receptor dimerization. Observed for pertuzumab.

D. For trastuzumab binding to domain IV prevents constitutive activation of ErbB2 via a number of mechanisms. Trastuzumab may also block domain IV contacts that are important for formation of heterodimers with some ErbB receptors (not shown).

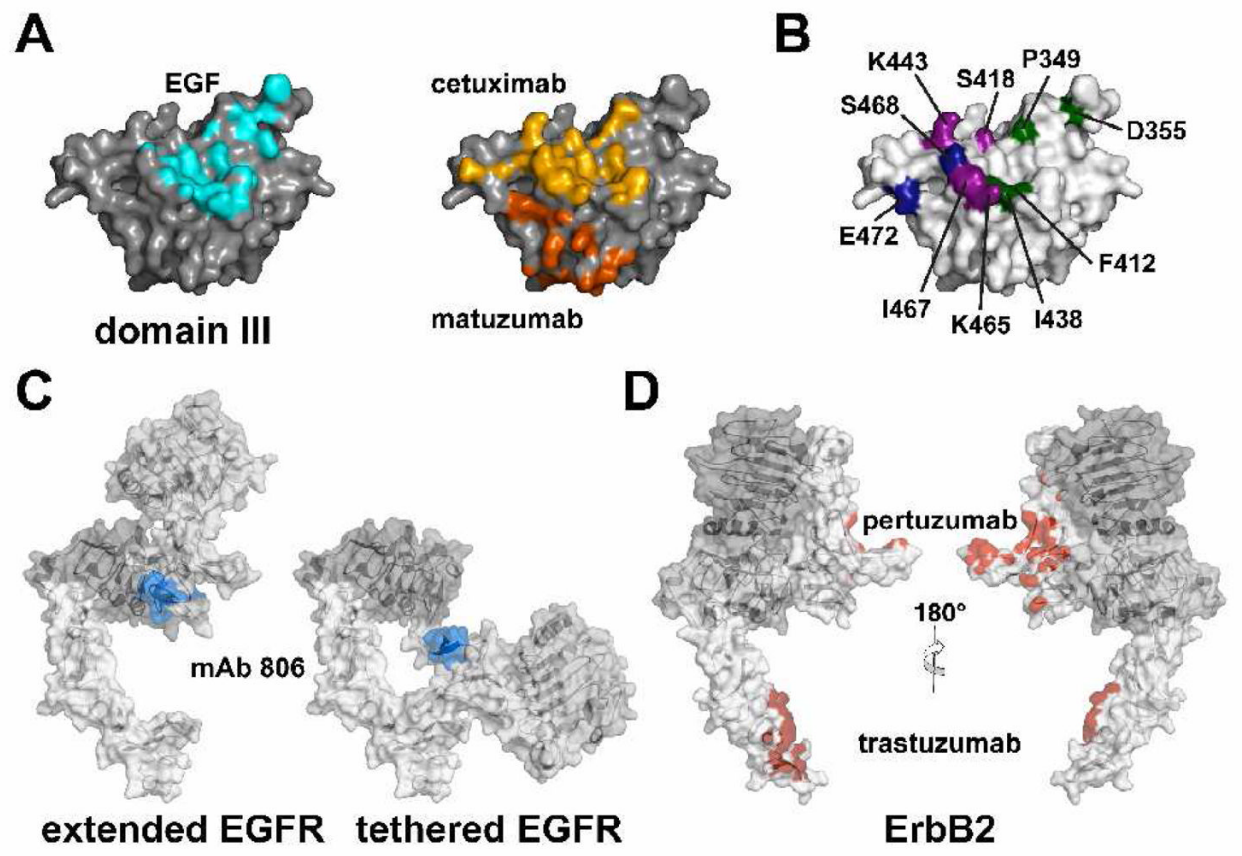

Figure 4. Epitopes for EGFR and ErbB2 antibodies

A. A molecular surface is shown of isolated domain III of EGFR looking down onto the ligand-binding site. Amino acids within 4 Å of the EGF (pdb id. 1ivo) are shaded in blue. The same surface is also shown with the cetuximab and matuzumab epitopes shaded in yellow and orange respectively.

B. The same projection of the domain III molecular is shown. Mutation of the highlighted amino acids affects binding of (i) panitumumab (green) [67], (ii) zalutumumab (magenta) [68] and (iii) mAb 13A9 (blue) [84].

C. The epitope of mAb 806 (blue) lies on a loop between C287 and C302 in domain II [83]. In both the extended ligand-bound, and the tethered structures of EGFR this epitope is partially occluded and would not be accessible for antibody binding.

D. The binding footprints (atoms within 4 Å of the bound Fab) of trastuzumab and pertuzumab are highlighted in red on a molecular surface representation of ErbB2.

Table 1

Properties of selected conformationally sensitive antibodies to the extracellular regions of ErbB receptors

mAb	Epitope information	Drug name[1]	Comments	Refs.[2]
EGFR				
1. Monoclonal antibodies derived from mice immunized with A431 cell EGFR (intact cells or membrane preparations)				
225	Domain III [65]	Cetuximab/Erbitux	Global approval for use against colorectal and head and neck cancers, wider clinical trials in progress	[11,12]
425	Domain III [66]	Matuzumab	Phase II trials	[37,38]
R1	Domain I/II [96]		Does not block ligand stimulated activation	[8]
2E9	Domain I [96]		Blocks low affinity cell surface EGF binding sites	[3]
108	Domain III [96]		Blocks high affinity cell surface EGF binding sites	[2]
13A9	Domain III [84]		Inhibits TGFα activation (and binding) of EGFR, but does not alter EGF binding or signaling	[97]
29.1	Carbohydrate		Used in purification of active EGFR	[98–100]
2. Monoclonal antibody derived from mice immunized with purified placental EGFR				
R3	Linear epitope on domain III (400–410) [101].	Nimotuzumab (TheraCIM)	Not conformationally sensitive, ongoing clinical trials, limited nation approval for head and neck cancer and glioma	[102]
3. Monoclonal antibody derived from rats immunized with the EGFR overexpression breast cancer cell MDA-MB 468				
ICR62	Group “C”, also binds EGFRvIII [103]		Phase I trials	[104]
4. Fully human monoclonal antibodies derived from transgenic mice immunized with A431-derivered EGFR				
ABX-EGF	Domain III [67]	Panitumumab/Vectibix	Approved for colorectal cancer, in trials for NSCLC	[48]
HuMax-EGFr	Domain III [68]	Zalutumumab	FDA fast track status for head and neck cancer trials	[52]
5. Fully human monoclonal antibody derived from library screening				
IMC-11F8	Domain III [64]		Binds to cetuximab epitope, phase I trials	[42,43]
6. Monoclonal antibody derived from mice immunized with fibroblasts expression EGFRvIII (also known as de2-7 EGFR)				
806	Domain II [83]		Binds to a fraction of wild-type receptor on EGFR overexpressing EGFR, chimerized to ch806, phase I trials	[56,58]
ErbB2/HER2				
Monoclonal antibody derived from mice immunized with NIH 3T3 cells that had been transformed with human ErbB2				
4D5	Domain IV [28]	Trastuzumab/Herceptin	Global approval from use in breast cancers	[61,105]
2C4	Domain II [63]	Pertuzumab/Omnitarg	In phase II/III trials for a range of cancers	[61,62]

[1] Antibody naming convention: ximab, chimeric; zumab, humanized; umab, fully human

[2] References here are limited to original articles describing basic, preclinical properties of the antibody.