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Review

Dynamic equilibrium between multiple active and inactive conformations explains regulation and oncogenic mutations in ErbB receptors

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Abstract

The ErbB growth factor receptor family members are key players in vital physiological and pathological processes. Like other receptor tyrosine kinases, the ErbBs are bi-topic membrane proteins, whose extracellular and intracellular domains are connected by single transmembrane span. In recent years the crystal structures of the extracellular and intracellular domains of some ErbBs have been determined. We integrated the available structural information with phylogenetic, biochemical, biophysical, genetic, and computational data into a suggested model for the regulation and activation of these receptors. According to the model, regulation is maintained by a dynamic equilibrium between monomeric and dimeric states in various conformations. Along this dynamic equilibrium, variations in the points of interactions within the dimers alter the activation state and ligand-binding affinities. The active state was recently shown to be associated with an asymmetric dimer of the kinase domains. That finding enabled us to elucidate, in molecular terms, the directionality observed in the activation process of ErbB heterodimers; it can explain, for example, the preferential activation of ErbB2 by ErbB1 over activation of ErbB1 by ErbB2. Sequence alterations that reverse this directionality lead to aberrant signaling and cancer. Our model also offers molecular interpretations of the effects of various oncogenic alterations that interfere with the regulatory mechanism.

Keywords: ErbB; EGFR; Molecular regulation; Pre-dimer; Functional and structural asymmetry

Contents

Abbreviations: CDK, cyclin-dependent kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; JM, juxtamembrane; MSA, multiple sequence alignment; NSCLC, non-small cell lung cancer; RTK, receptor tyrosine kinase; TM, transmembrane; wt, wild type

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1. Introduction

The four ErbB growth factor receptors are members of one of the most studied protein families in cancer research, also known as the HER family. ErbB1, the first member to be discovered, is also known as the epidermal growth factor receptor (EGFR). The ErbB family is ubiquitously distributed throughout the animal kingdom [1], and plays an essential role in vital cellular processes such as proliferation, differentiation, migration, and apoptosis [2,3] as well as in various pathologies [4].

The ErbBs belong to the receptor tyrosine kinase (RTK) super-family [5], all of which are transmembrane (TM) proteins that are activated by the binding of extracellular ligands, such as the epidermal growth factor (EGF). Structurally they consist of an N-terminal extracellular ligand-binding domain, a single TM span, and a large intracellular domain that includes the catalytic kinase domain and a regulatory C-terminal domain (Fig. 1). Activation of the ErbBs induces phosphorylation of tyrosine residues that are located in the receptor's intracellular domain and serve as docking sites for other proteins, thereby allowing transfer of the signal into the cell. The complex signaling network of the RTKs in general, and of the ErbB family in particular, has been the focus of intensive research (see, e.g., [3,6,7]).

Interestingly, the second family member, ErbB2, lacks the capacity to bind ligands, and the third, ErbB3, has an inactive kinase [8]. Both are nevertheless vital to embryonic development, as are the EGFR and the fourth family member, ErbB4. This apparent paradox is explained by the fact that the basic functional unit in the signaling of the ErbB family, as in other RTKs, is a dimer. Thus, ErbB2 and ErbB3 operate by combining with other ErbBs to form heterodimers [3,9]. Phosphorylation of ErbB3 by its partner leads to a specific and unique signaling pathway, explaining the significance of ErbB3 despite its being catalytically defective.

The ErbBs mediate an essential cellular signaling network; consequently, their activation is subject to several layers of regulatory control [10]. We will focus on the molecular details of the regulatory mechanisms that operate within the ErbB proteins. The regulatory role played by the extracellular and TM domains of the ErbBs has been extensively discussed. In contrast, most aspects of the regulatory mechanisms located in the intracellular domain have only recently been elucidated. Here we discuss the new developments and suggest a model of EGFR regulation, presented in Fig. 1, which integrates the multiple layers of control imposed by the various structural elements of this protein. The model is based on our two previous publications pertaining the regulatory roles of the TM and intracellular domains of the EGFR [11,12]. We are now able to incorporate additional information from a recently published crystal structures and biochemical experiments. The current model illustrates a mechanism of dynamic equilibrium, which is manifested by interconversions between different conformations of the receptor in the ligand-free state. Binding of ligand disrupts this equilibrium and invokes a consecutive process that triggers activation. In the first section of the review, we explain the regulation imposed within and between the receptor's different domains, which is illustrated in Fig. 1 and summarized in Section 3. Differences and similarities in regulatory mechanisms among the various ErbBs are elaborated in Section 4. In the fifth section, by referring to the regulatory model and an evolutionary conservation analysis of ErbBs from various species, we predict the molecular effect of cancer-causing mutations in the ErbBs. We note that the mechanisms of regulation of the EGFR might depend on the cell type and the stage of the cell cycle [7]. We do not cover processes that control ErbB functions at the cellular level, such as down-regulation, degradation, recycling, endocytosis, and intracellular trafficking, as these are extensively discussed [3,4,6]. Other aspects of EGFR regulation were reviewed in [7,13–16].

2. Mechanism of regulation in the ErbB family

The general scheme of the activation mechanism in RTKs involves binding of ligand to the extracellular domain, which leads to dimerization of the receptor followed by structural

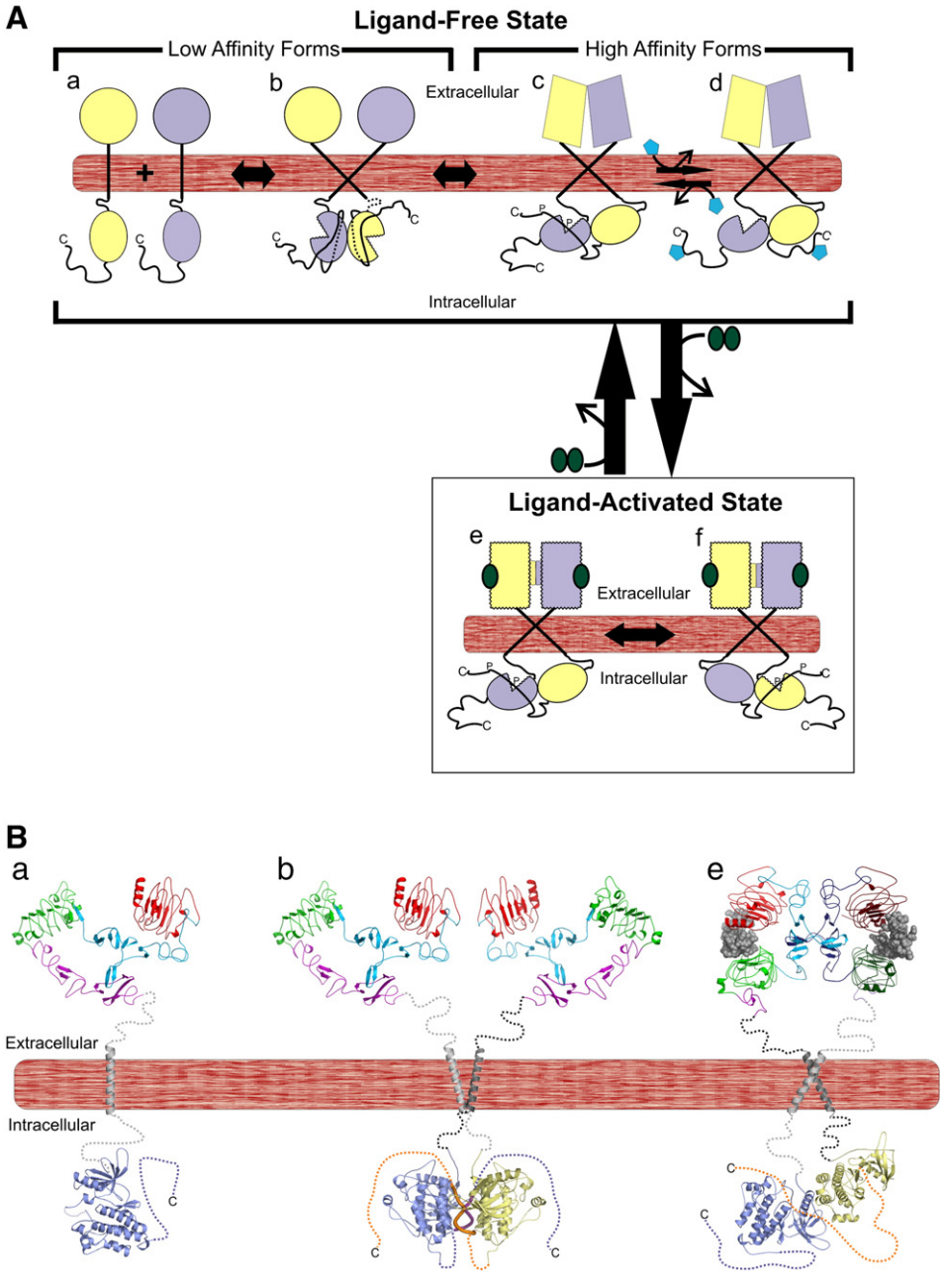
changes and phosphorylation of tyrosine residues in its intracellular domain [5]. Accumulating new evidence has resulted in fundamental extensions and modifications to the above basic mechanism in the ErbBs. These modifications are reviewed here.

2.1. Regulation by dimerization

There are growing evidences that at least fraction of the ErbBs are clustered in the membrane, and random collisions that could lead to the formation of dimers and other oligomers are therefore very likely to occur [7,17–21]. As a result, it is anticipated that another layer of regulation, in addition to ligand-binding, is needed in order to prevent activation induced by random dimerization. For example, the formation of stable inactive dimers or oligomers could serve as a mechanism for the required control. Studies have indeed shown that although dimerization (which requires spatial proximity between two ErbBs) is a necessary step toward ligand-induced activation, such proximity is not in itself sufficient to promote activation [7,20,22–27].

Because the RTKs are multi-domain proteins, the term 'dimerization' should be used with caution, i.e., with implicit relevance to the location of interaction. As an example, mediation of contacts by the extracellular domain does not necessarily mean that the intracellular kinase domains directly interact, and vice versa. The location of contact formation between the two subunits has crucial implications for the activation state of the receptor (see Fig. 1).

As mentioned above, numerous studies have shown that the EGFR can form ligand-independent dimers, or 'pre-dimers'



[20–29]. Although some studies suggested that the percentage of EGFR oligomers on the cell surface is low in comparison to the monomers [19,29], a recent study demonstrated that, in the absence of ligand stimulation, the majority of EGFR and ErbB2 form dimeric structures on the cell surface in single live cells that express physiological levels of the receptors [20]. This result, which was obtained using a novel combination of two methods, single wavelength fluorescence cross-correlation spectroscopy (SW-FCCS) and Förster resonance energy transfer (FRET), is presumably most relevant to the physiological situation. EGF stimulation leads to additional and significant receptor clustering on the membrane [20].

The pre-dimers are probably inactive [7,22,28]. However, the nature of the specific interface within the dimers detected in those studies has yet to be fully elucidated. There are indications that all ErbBs display ligand-independent contact formation between their TM domains [25]. Recent work points out that the interactions between the TM domains are extremely modest [30]. However, these studies were conducted in micellar solutions and their relevance to the cellular environment is questionable. In addition to possible interactions within the TM domains, there is evidence that the cytoplasmic domain [22], and even more specifically, the kinase domain [23], are necessary for the formation of at least some of these ligand-independent dimers.

Binding of EGF to the pre-dimers on the cell surface displays much higher affinity than to the monomers [29] (see Section 2.5). It was suggested that pre-dimer formation helps facilitate the formation of active dimers, which is not limited by diffusion along the plasma membrane, even at low EGF concentrations [29]. Moreover, clustering of ErbBs in the membrane may contribute to a rapid spread of the signal through receptors that are not bound to the ligand, but are activated by their adjacent active dimers [7].

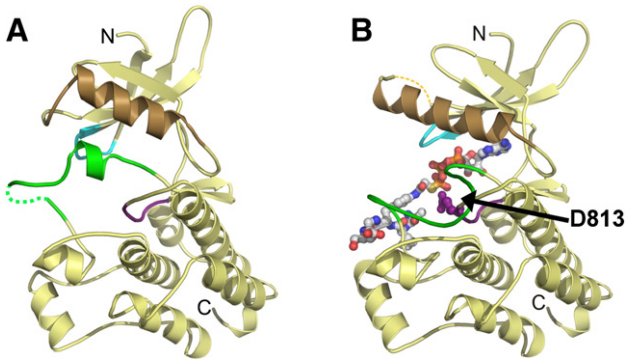


Fig. 2. Regulatory elements in the kinase domain. The kinase domain of the EGFR (residues 685−957) is depicted in a yellow ribbon representation, in its inactive (A) (PDB entry — 1gs7 [34]) and active (B) (PDB entry — 2gs6 [34]) conformations. Regions that are not ordered in the crystal structures are depicted by dashed lines. Regulatory elements are depicted by different colors: the activation loop (residues 831−852) in green, the αC-helix (residues 729−745) in brown, the phosphate-binding loop (residues 695−700) in cyan, and the catalytic loop (residues 812−818) in purple. In panel B, the catalytic residue Asp813 (in purple) and the ATP-analog–peptide conjugate (colored by atom type: carbon in white, nitrogen in blue, oxygen in red, phosphate in orange, and sulphur in yellow) are displayed in a balls-and-sticks representation.

2.2. Regulation by the intracellular domain

The kinase domain, which is located in the intracellular domain, is the catalytic unit in all RTKs. It is comprised of two subdomains, known as the N-terminal (N-) and the C-terminal (C-) lobes (Fig. 2). The phosphate donor (ATP), as well as the substrate designated for phosphorylation, binds in a cleft flanked by the two lobes. The ATP is held in position by a phosphate-binding loop (P-loop), which is located at the N-lobe. The substrate is positioned near the ATP's phosphates by the

Fig. 1. Regulation of the EGFR. The EGFR is represented by its main structural elements, namely the extracellular part, the TM domain, and the intracellular part comprising the kinase and C-terminal domains. Location of the membrane is marked by the brown bar. Panel A presents a scheme of the regulatory mechanism maintained by interconversion between multiple forms. We note that all forms in the ligand-free state are in dynamic equilibrium with one another. Two monomers of the EGFR are displayed, colored yellow and purple. Panel B displays a ribbon representation of crystal structures available for specific domains of the EGFR. The fragmentary structures are schematically combined here to illustrate the entire receptor in a few conformations that correspond to panel A. Regions for which a crystal structure is not available are depicted by dashed curves. The crystal structures of the extracellular domain are colored according to its subdomains (I in red, II in cyan, III in green and IV in magenta; for the dimer in form 'e', one of the monomers is in darker shades for clarity). The EGF ligand is represented by gray space-filled atoms. The TM helices are colored in two shades of gray. The kinase domains are colored in yellow and purple as in panel A. A fragment from the C-terminal domain that is available in the crystal structure is displayed as a tube, colored in darker shades of the color of the kinase domain on the same monomer. The ligand-free state is characterized by interconversion between many different conformations, while the scheme in panel A depicts only representative forms. In both panels, form 'a' stands for a monomeric conformation in which the kinase domain is inactive (residues 679−964; PDB entry — 2gs7 [34]). Form 'b' illustrates a dimeric form demonstrating the formation of an inactive intracellular complex (residues 672−995; PDB entry — 1m17 [32]), and the TM pair in the inactive state (the coordinates were taken from [11]). The extracellular domains in forms 'a−b' are predicted to alternate between tethered and extended conformations that are embodied by an abstract conformation in panel A. The crystal structure of the extracellular domain (forms 'a−b' in panel B) depicts only the tethered conformation in the ligand-free state (residues 2−614; PDB entry — 1YY9 [64]). Forms 'a−b', in which the extracellular domains are separated, are predicted to have a relatively low ligand-binding affinity. Based on experimental data (see main text) we predicted that an intracellular asymmetric dimer is one component of the high-affinity state. Forms 'c−d' display a dimeric conformation in which contact formation is mediated by the TM, the extracellular, and the intracellular domains. In these forms the extracellular domains assume an extended conformation allowing contact, which is likely to significantly increase the binding affinity of the receptors for the ligand. No structural information is available for such conformations of the extracellular domains, which are probably transient. The intracellular asymmetric dimer is comprised of activating (yellow) and activated (purple) monomers, which can potentially switch orientations (as illustrated in forms 'e−f'). The equilibrium between forms 'a', 'b' and 'c' is dependent on their inherent stability, while conversion to form 'd' is dependent on an unknown external factor that is depicted by a cyan polygon. We emphasize that the conformation assumed by form 'd' is entirely hypothetical. Binding of the growth factor ligands, depicted by green ovals in panel A (or gray spheres in panel B), induces conformational changes in the extracellular domains, including exposure of the 'dimerization arm' illustrated in forms 'e−f'. The ligand-bound extracellular domains (residues 2−512; PDB entry — 1IVO [68]) are in extended conformation, and allow contact formation between the two monomers. In this ligand-activated state the TM complex assumes the active conformation (the coordinates were taken from [11]). The kinase domains within the intracellular asymmetric dimer (residues 669−967; PDB entry — 2gs6 [34]) can potentially switch orientations, such that the activating and activated monomers exchange roles (forms 'e−f'; panel B illustrates only the structure corresponding to form 'e').

'activation loop' located in the C-lobe. In most RTKs the activation loop assumes a closed conformation in the inactive state, thereby preventing substrate binding. Activation involves conformational changes and phosphorylation of the activation loop [10,16,31]. These events are coupled to a concurrent movement of another regulatory element, the αC-helix located in the N-lobe, into a final conformation that is catalytically competent. The actual catalytic process is performed by the highly conserved catalytic loop in the base of the active site located in the C-lobe. Catalysis requires that all the above regulatory elements are positioned in the proper spatial arrangement.

Until recently, it was thought that the constant state of the kinase domain of the EGFR and other ErbBs is the active conformation. This assumption was based on structural data, which showed that although the activation loop is not phosphorylated, all the regulatory elements of the EGFR's kinase domain are ready for catalysis [32]. That structure concurred with previous biochemical data showing that activation of the ErbB family is independent of their phosphorylated state [33]. Subsequently, when the crystal structure of the EGFR in complex with the large inhibitor GW572016 was determined, a putative inactive conformation of the kinase domain was observed. It was suspected, however, that this conformation was induced by the bulky inhibitor [12,34,35].

In contrast to the supposedly constant active conformation, biochemical data showed that the EGFR is not constantly active in cell membranes [5]; indeed, aberrant conditions (such as mutations in the EGFR) that lead to ligand-independent activation are known to be related to cancer. Thus, the phosphorylation-independent activation of the EGFR [33], as well as the lack of conformational regulation observed in the first crystal structure [32], prompted investigators to seek regulatory elements that govern the inhibition of the EGFR within the intracellular domain [12,34,36].

2.2.1. The inactive state

In a recent study by Zhang and his co-workers [34] it was shown that the kinase domain of the EGFR indeed also possesses an inherently inactive conformation. Those authors demonstrated that the constitutive active conformation of the EGFR that was seen in the first crystal structure [32] was induced by intermolecular contacts within the crystal. A mutation in the kinase domain that disrupted such contacts led to the identification and determination of an inactive kinase conformation [34], which was very similar to the GW572016-bound structure [35].

The inactive conformation of the EGFR resembles those of the cytoplasmic tyrosine kinase Src and the serine/threonine cyclin-dependent kinases (CDKs) [34]. In this state the αC-helix, together with the loop preceding it, forms contacts with the N-terminus of the activation loop, combining this threesome in an inactive conformation (Fig. 2A). Consequent displacement of the αC-helix and the activation loop prevent the proper positioning of catalytically important elements (Fig. 2B). Any interference with the packing of the activation loop against the αC-helix and its preceding loop, caused for example by mutations, would lead to increased auto-kinase activity and cell transformation [37]. These mutations will be discussed in Section 5.

2.2.1.1. The C-terminal domain serves as an auto-inhibitor via direct contacts with the kinase domain within a dimer. By and large, ErbB signaling and part of its regulation are mediated via the C-terminal domain. Following activation, phosphorylated tyrosines on the C-terminal domain serve as docking sites for the subsequent proteins in the signaling cascade, as well as for regulatory proteins that control processes such as internalization and degradation [3,4,38]. We recently suggested that a fragment from the C-terminal domain of the EGFR, immediately following the kinase domain, serves as an inherent auto-inhibitor of the receptor via its direct contacts with the kinase domain [12]. Other studies suggested that the same fragment in the C-terminal domain binds to the juxtamembrane (JM) domain via electrostatic interactions, and this contact mediates receptor dimerization [39].

The structures of the intracellular domain of the EGFR contain the kinase domain (residues 672–957; amino-acid numbers throughout the text are based on mature EGFR) and a fragment from the C-terminal domain (residues 958–995) [32,34,35]. According to our earlier analyses, the kinase domain of the EGFR displays two surface patches of positive electrostatic potential, one in the N-lobe and the other in the C-lobe, both located on the back side of the active site [12]. On the other hand, the C-terminal fragment that is present in the structure (residues 979–995) is highly negatively charged.

The structures of the wild-type (wt) EGFR kinase domain reveals a crystallographic symmetric dimer in which contacts between the two kinase domains are mediated by the two C-terminal fragments (Fig. 3) [32,34]. Within this dimer, each

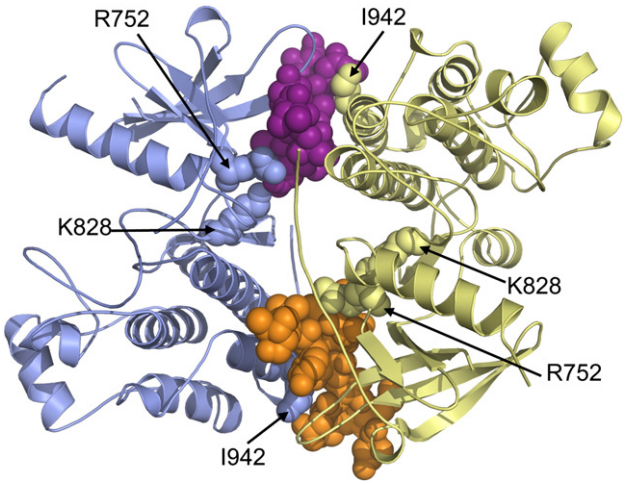


Fig. 3. The symmetric inactive dimer of the kinase domain. Within the inactive symmetric crystallographic dimer, contacts between the two kinase domains (residues 672–966; depicted in yellow and light purple ribbon representations) are mediated by their following two C-terminal fragments (residues 982–964, depicted as space-filled atoms and colored in darker shades of the color of the kinase domain on the same monomer). Residues on the kinase domain that are in contact with the C-terminal fragment, and whose replacement was implicated in heightened basal activity (Lys828 and Ile942) or was found in cancer cells (Arg752), are depicted as space-filled atoms. Coordinates were taken from PDB entry — 1gs2 [34]. The biological relevancy of this crystallographic dimer is discussed in Section 2.2.1 above.

kinase domain contacts the two C-terminal fragments via its two positively charged surface patches. In addition to this charge complementarity, our previous analyses showed that the areas that are buried between each kinase domain and the two C-terminal fragments in this complex are extremely large (1419 Å and 1048 Å for the N- and C-lobe interfaces, respectively) [12]. Moreover, although the two kinase domains in the complex barely interact directly, they are connected via the C-terminal fragments through a network of salt bridges and hydrogen bonds [12]. Overall, based on the above computational analyses [12], this crystallographic complex appears to be stable. We also observed that one of the phosphorylation sites on the C-terminal domain (Tyr992) is buried within the complex and is inaccessible to phosphorylation [12]. Thus, we speculated that this complex represents an inactive form of the receptor (form 'b' in Fig. 1). As mentioned in Section 2.1, there are experimental indications for the existence of ligand-independent dimers, and that the cytoplasmic domain is involved in the contact formation [22,23]. We therefore suggested that the complex seen in the crystal structure might represent this ligand-independent dimer. The apparent stability of the crystal complex bears the potential to prevent ligand-independent activation induced by random contacts (see Section 2.1).

In accordance with the above model supporting the role of the C-terminal domain as an auto-inhibitor of the EGFR, variations in the C-terminal domain in naturally occurring retroviral oncogene variants (v-ErbB) are associated with malignant diseases because of their increased rate of auto-phosphorylation [40–46]. More specifically, engineered deletions or mutations in the C-terminal fragment in contact with the kinase domain in the crystal structure display heightened catalytic activity [34,38]. We predict such variations to cause destabilization of the inhibitory interactions between the kinase and the C-terminal domains, promoting activation [12]. Furthermore, specific replacements of four negatively charged residues on the C-terminal fragment (residues 979−982) by their polar equivalents resulted in higher auto-kinase activity and partial transforming phenotype [38], supporting the role of charge complementarity in the inhibition by the C-terminal domain. Correspondingly, substitution of alanine for the positively charged Lys828 (K828A), which participates in intramolecular interactions with the C-terminal domain (Fig. 3), resulted in higher basal activity of the receptor [34]. Interestingly, this residue participates in the abovementioned polar network of interactions that mediates the contacts within the symmetric dimer [12]. These results imply that the C-terminal domain indeed participate in inhibition via direct interactions with the kinase domain.

It was previously suggested that the monomeric inactive state of the kinase domain is stabilized by direct contacts between the kinase and the C-terminal domains [34,35]. However, *in vitro* experiments, conducted using a truncated form of the intracellular domain of the EGFR (residues 672−998) at a low concentration that mimics the monomeric form of the receptor, showed that the catalytic activity of the kinase domain is not altered by further truncation of the C-terminal fragment (residues 965−998) [34]. This implies that the C-terminal fragment does not participate in stabilizing the monomeric inactive conformation. On the other hand, at a high concentration of the EGFR that corresponds to the concentration at which dimers are formed, truncation of the C-terminal fragments greatly increased the inherent activity of the kinase domain [34]. Evidently, therefore, the effect of the C-terminal fragment is manifested only when the EGFR forms dimers. Interestingly, a mutation (I942E) in a residue which, according to the symmetric crystal dimer, contacts the C-terminal fragment of the adjacent monomer (Fig. 3), led to higher basal activity of the receptor [34], supporting the biological relevance of this dimer and its role in auto-inhibition.

Overall, according to our model, the inactive state of the EGFR involves an intracellular dimer that might resemble the symmetric dimer seen in crystals. This crystallographic complex, however, evidently does not play a role in the active state, as mutations in residues located on its interfaces did not alter the ligand-induced kinase activity of the EGFR [34].

2.2.2. The active state

The exact conformation of the active state of the intracellular domain, after eluding investigators for years, was finally revealed by Zhang and his co-workers [34]. The crystal lattice of the EGFR includes, in addition to the symmetric dimer discussed in Section 2.2.1, an asymmetric dimer [32,34]. Through a series of comprehensive and elegant experiments, Zhang and his co-workers [34] showed that this asymmetric dimer represents the active form of the intracellular domain, in which one kinase activates its partner (forms 'e−f' in Fig. 1). Interestingly, the involvement of an asymmetric dimer in regulation was predicted a decade ago by Groenen et al. [47].

This asymmetric dimer closely resembles the complex between cyclinA and activated CDK2 [34] in which cyclinA serves as an activator of CDK2 [48]. In the asymmetric dimer of the EGFR, one kinase maintains contact with its partner through the C-lobe in a way that mimics the role of cyclinA. Thus, by analogy, we refer to it as the 'activating' monomer (the yellow kinase in form 'e' in Fig. 1). The second kinase, which forms the dimeric interface via its N-lobe, mimics CDK2, and we therefore refer to it as the 'activated' monomer (the purple kinase in form 'e' in Fig. 1). Interaction via the N-lobe leads to displacement of all the regulatory elements into the catalytically active conformation.

2.3. Regulation by the cytoplasmic juxtamembrane domain

The region immediately following the TM domains, termed the JM domain (residues 645−672), also plays a role in regulation [36,39,49]. An EGFR mutant that sustained a deletion of the JM segment (residues 645−657) displayed similar basal activity to that of the wt EGFR, but lost its ability to interact with a second EGFR molecule and to undergo ligand-induced phosphorylation [39]. Accordingly, this region can be expected to be involved in the active dimeric state, but not in auto-inhibition. Nevertheless, there are evidences that phosphorylation of Thr654 and Thr669, both located on the JM domain, by protein kinase C/D (PKC/D), inhibits some of the signal transduction cascades followed by the activation of the EGFR [50–52]. This suggests that the JM domain has the potential to play a role in inhibition.

The JM segment carries a positive net charge and was therefore suggested to bind to a negatively charged fragment on the C-terminal domain (residues 979–991) [39], or, alternatively, to the negatively charged inner leaflet of the membrane [36]. It was further suggested that binding of the negatively charged Ca^{2+}/calmodulin complex would repel the JM domain from the membrane, with subsequent possible rearrangement of the kinase domain into an active state [36,49,53]. Initial activation of the EGFR leads to a transient increase in the cellular level of free Ca^{2+}, thereby activating the Ca^{2+}/calmodulin complex [36,49]. Taken together, therefore, these studies suggested that binding of the Ca^{2+}/calmodulin complex to the JM domain might serve to amplify the initial signal by subsequently activating large numbers of additional receptors.

There are pieces of evidence that phosphorylation by PKC or glutamate substitution of Thr654 can block the binding of calmodulin to the EGFR [54,55], while, conversely, binding of calmodulin blocks phosphorylation by PKC [54]. Thus, the addition of a negative charge on Thr654 probably electrostatically interferes with calmodulin binding. On the other hand, the addition of a negative charge on Thr699, also located on the JM domain, did not affect the binding of calmodulin [54]. Overall, the mechanisms by which the JM domain contributes to the regulation of the EGFR still have to be determined.

2.4. Regulation by the TM domain

The TM domain of the ErbBs is more than a passive peptide anchoring the receptors to the membrane; it also serves as an additional layer of regulation [56]. The ErbB TM domain has an inherent tendency to associate in the membrane in two different forms that are mediated by two different dimerization motifs [11,25]. Based on energy considerations, our earlier computational analysis assigned these dimerization motifs in ErbB2 to the inactive (form 'b' in Fig. 1) and active (forms 'c−f' in Fig. 1) states and suggested that the TM dimers could switch between these two states by rotating through 120° in a screw-like motion [11,57]. This and other studies suggested a rotation-coupled activation mechanism in which ligand binding induces a rotational change in the TM helices from the inactive to the active conformation [11,58]. The change in conformation is translated into reorientation of the intracellular kinase domains, leading to activation (Fig. 1) [11,12,26,27,58]. This molecular-switch model provided an explanation, at the molecular level, for known ErbB2 mutants or naturally occurring variants [11]. For example, the V664E oncogenic mutation in the rat ErbB2 (*Neu*) [59], which is located within one of the dimerization motifs, was predicted to induce a shift toward the active conformation and hence toward enhanced activation [11]. It is noteworthy that although a similar substitution in the human ErbB2 showed elevated ability to transform cells [60], this variation in ErbB2 was not detected in human cancer cells, probably since it requires the substitution of 2 bp. Nevertheless, another mutation (Ile654Val) in the TM domain of ErbB2 was clinically shown to be associated with an elevated risk for breast cancer [61]. We note that a bias, presumably negligible, resulting from the presence of another mutation, was later reported in the genotyping of this ErbB2 variant [62]. The observed existence of oncogenic mutations or variations in the TM domain of the rat and human ErbB2 [56,59,61,63] further supports a regulatory role for this domain.

It is interesting to note that in the predicted inactive conformation, the TM segments interact through the dimerization motif located at the C-terminal part of the helix, resulting in close proximity of its intracellular ends (~9 Å), as illustrated in form 'b' in Fig. 1. Rotation to the active state, mediated by the N-terminal dimerization motif (forms 'c−f' in Fig. 1), would impose a larger distance (~19 Å) between the cytoplasmic ends of these helices [11]. Correspondingly, the N-termini of the putatively inactive intracellular dimer are much closer to each other (~20 Å; form 'b' in Fig. 1B) than in the active asymmetric dimer (~50 Å; form 'e' in Fig. 1B). In addition, switching of the TM domains to the active state brings their extracellular ends closer to one another. This is probably followed by contact formation between the extracellular domains (forms 'c−f' in Fig. 1). Thus, in accordance with the rotation-coupled activation mechanism, the activation state of the TM helices allows leverage that controls the positions of the intracellular and extracellular domains.

2.5. Regulation by the extracellular domain

In recent years, a growing body of structural information has led to substantial progress in understanding the mechanisms of ErbB regulation by their extracellular domains. The ligand-free structures of the extracellular domains of EGFR [64], ErbB3 [65], and ErbB4 [66] display a tethered auto-inhibited state, while the EGFR's ligand-bound extracellular domain [67,68] shows an extended conformation that allows contact formation between two subunits (Fig. 1B). Interestingly, the 'orphan' (ligand-less) receptor ErbB2 resembles the ligand-bound active conformation of the EGFR [69,70].

The role of the extracellular domain in regulation of the ErbBs, excluding ErbB2, is to impose a ligand-mediated activation [7,71]. Indeed, truncation of the extracellular domain, as seen in viral ErbB variants that are related to oncogenic transformation, leads to ligand-independent activation of the receptors [42]. These findings also suggest that other domains, namely the TM and intracellular parts, possess an inherent ability to interact and to become activated.

The structure of the extracellular domain has been described in detail in a few comprehensive reviews (e.g., [15,71]). In brief, it comprises four subdomains, termed I–IV (Fig. 1B). In the inactive conformation, subdomains II and IV interact to lock the structure of the extracellular domains of ErbB-1, -3, and -4 in a tethered, putatively inactive conformation [72] (forms 'a−b' in Fig. 1B). Biochemical experiments indicate, however, that other elements in the extracellular domain are probably also involved in the auto-inhibition [73,74]; thus, the available structures do not yet provide the whole story of inactivation [7]. In the extended dimeric ligand-bound conformation, each growth factor ligand binds one extracellular domain through subdomains I and III [67,68] (form 'e' in Fig. 1B). Contacts between the two EGFR extracellular domains are mediated solely by the receptors, mainly

by a loop (the 'dimerization arm') in subdomain II [68]. Mutations in this loop prevent ligand-induced activation [67,68].

Measurements of the binding affinity of EGF to its receptor pointed to the existence of two different EGFR populations on the cell surface, each with its own ligand-binding affinity [7,71]. It was proposed that 92−95% of the receptors exhibit low-affinity binding, with a K_d of approximately 6−12 nM. These receptors were predicted to assume the tethered conformation of the extracellular domain, in which the two subdomains responsible for making contact with the ligand are far apart, allowing the ligand to make contact with only one of two interfaces [65,71,72]. Some 2−5% of the receptors were proposed to bind EGF with high affinity, with a K_d below 0.1 nM, and these were considered to assume the extended conformation that brings the two ligand-binding subdomains closer [29,65,71,72]. However, mathematical models describing the kinetics of interactions between EGF and its receptor showed that there is no direct correlation between the conformations seen in the crystal structures and the two different binding affinity populations [75]. Recent findings suggested that the low-affinity population actually corresponds to an ensemble of conformations that are related to interconversion between the tethered and the extended forms of the extracellular domain [74]. The conformation of the high-affinity state is less well characterized and is thought to involve additional elements, such as other proteins [76,77] (form 'd' in Fig. 1A) as well as cell regulatory mechanisms such as endocytosis and degradation [75]. Moreover, the high-affinity binding is totally dependent on specific regions within the intracellular domains [74,77,78] (see also Section 2.6).

Overall, it was suggested that the low- and high-affinity binding modes, which appear macroscopically as two distinct populations that do not interact, rather represent interconversion between multiple conformations [77]. Some of these conformations are presented in the dynamic equilibrium presented in Fig. 1, where each form displays its own distinctive binding affinity and specific probability, based on its inherent stability. Conformations that display lower affinity for the ligand are presumably more stable energetically than the higher affinity conformations; this would explain the above macroscopic estimation that the binding affinity of 92−95% of the receptors is low. Nevertheless, the ligand preferably traps the less stable, high-affinity conformations. In the high-affinity state, the specific orientation of the TM and intracellular domains may trigger contact formation between the monomers' extracellular domains (forms 'c−d' in Fig. 1A), although not necessarily in exactly the same manner as in the ligand-bound dimer (forms 'e−f' in Fig. 1A) [56]. Accordingly, a recent single-molecule analysis of EGF binding on the surface of living cells predicted that the macroscopic high-affinity population is represented by pre-dimers, which bind EGF at a rate two orders of magnitude higher than that of binding by the monomeric receptor [29].

As a result of ligand binding, the formation of contacts between the two monomers is further stabilized, thereby shifting the equilibrium toward the ligand-activated state [29,71] (forms 'e−f' in Fig. 1A). Recent findings predicted the formation of a kinetic intermediate following binding of the first EGF and prior to binding of the second [29]. Thus, binding of one EGF to the pre-dimer may induce allosteric conformational changes that enhance the binding of the second EGF, hence displaying positive cooperativity [29].

Somatic or engineered alterations in the extracellular domain can potentially shift the equilibrium (depicted in Fig. 1) by thermodynamically stabilizing or destabilizing specific forms, thereby modifying the macroscopically measured ligand-binding affinity. For example, interference with the tethered conformation by mutations may lead to a higher average of the low-affinity binding mode by shifting the equilibrium toward the extended conformations [65,72]. On the other hand, alterations in the dimerization interface in the extracellular domain could destabilize contact formation and weaken the interaction between the EGFR and EGF, eventually lowering the inherent binding affinity [73].

2.6. Interactions within the intracellular domains affect ligand-binding affinity

As suggested in Section 2.5, the macroscopically measured low-affinity mode of binding of ligand to the EGFR is actually an average of the binding affinities of few different conformations that undergo interconversion. Interestingly, this average binding affinity could be modulated by intracellular elements, probably through shifts between conformations in the equilibrium scheme. For example, deletion of a fragment from the C-terminal domain (residues 984−996) resulted in an increase in the average binding affinity of the low-affinity mode by approximately threefold [78]. A similar effect was reported for a Y992E mutation within this region [77]. The same C-terminal fragment mediates the putatively inactive dimer (Fig. 3) discussed in Section 2.2.1. We previously suggested that deletion of this fragment would destabilize the dimer [12] (form 'b' in Fig. 1), allowing more abundant ligand-independent formation of the asymmetric intracellular dimer (forms 'c−d' in Fig. 1A), which displays high-affinity binding. Overall, the effects of the mutations would result, on average, in higher binding affinity [77,78].

The macroscopically measured high-affinity mode, representing only 2−5% of the receptors, may reflect thermodynamically unstable high-affinity conformations that undergo interconversion with the more stable low-affinity forms. A clue to the conformations of the high-affinity state comes from engineered alterations to the receptors, where truncation of the entire intracellular domain resulted in the complete loss of high-affinity binding [74,79]. More specifically, two regions were described as particularly crucial for high-affinity binding, namely the segment comprising residues 921 to 940, and the last 63 residues at the C-terminal end of the protein. Deletion of either of these regions completely abolished the high-affinity binding mode [78,79]. It was suggested that the C-terminal end of the intracellular domain might comprise a binding site for external regulators that stabilize a high-affinity state [77] (form 'd' in Fig. 1A). Deletion of this region would prevent the formation of this high-affinity form. Interestingly, we note that the other segment related to high-affinity binding (residues 921−940) comprises a large part of the interface within the active dimer. That finding led us to suspect that at least some of the high-affinity conformations could be correlated with the formation of the active asymmetric intracel-

lular dimer, as represented by forms ‘c–d’ in Fig. 1A. We suggest that deletion of this region might disrupt the active intracellular dimer, thereby shifting the dynamic equilibrium toward lower affinity forms. This model raises a question: does part of the high-affinity class (form ‘c’ in Fig. 1A) represent a ligand-independent active state that might be responsible for the basal activity of the receptor?

It should be emphasized that the high-affinity state is still only poorly understood and that not all of the experimental results obtained to date in this field can be satisfactorily explained. Accordingly, the high-affinity forms presented in Fig. 1 are provisional and presumably represent only a fraction of the conformations comprising the high-affinity state.

3. A multilayered model for the regulation of EGFR activity

Research on EGFR regulation recently reached a critical stage, at which the accumulated experimental data and models are converging in support of a unified mechanism of receptor activation. In this scheme, each of the domains in the EGFR constitutes an additional level in the regulatory mechanism. In the ligand-free state the receptors shift between multiple monomeric and pre-dimeric forms. In the monomeric form, the kinase domain assumes an inherently inactive conformation that prevents catalytic activity (form ‘a’ in Fig. 1). In the dimeric form, the TM helical segments preferentially interact through the dimerization motif located at the C-terminal part of the helix, resulting in close proximity of the intracellular ends to one another, but a substantial distance between the two extracellular ends (form ‘b’ in Fig. 1). In this conformation the two intracellular domains interact with one another, for example, they form the inactive crystallographic dimer in which the N-termini are close to each other while the extracellular domains are separated. This dimeric conformation is catalytically inactive. In both the monomeric and the dimeric inactive states (forms ‘a–b’ in Fig. 1) the extracellular domain might assume either tethered or extended conformations that undergo interconversion, each displaying its distinctive affinity for the growth factor ligands.

A small energy barrier separates the stable inactive form from the less stable active form of the TM dimer, mediated by the C- and the N-terminal motifs, respectively [11]. Thus, we assume that, while in the ligand-free state, the active TM dimer could be populated to some extent in which the cytoplasmic ends of the monomers are far apart and the extracellular ends are close together (forms ‘c–d’ in Fig. 1A). This would lead to the destabilization of the inactive intracellular dimer, prompting the formation of the active asymmetric intracellular dimer in which the N-termini are distant from one another, and hence possibly to transient activation of the receptor (form ‘c’ in Fig. 1A). This state could also be stabilized by binding of regulatory proteins (form ‘d’ in Fig. 1A). At the same time, the close proximity of the extracellular ends of the TM segments would lead to contact formation between the extracellular domains. These conformations would typify the high-affinity ligand-binding mode.

According to the model, the ligand-free state is characterized microscopically by dynamic equilibrium between different conformations (forms ‘a–d’ in Fig. 1). Because the low-affinity forms (‘a–b’) possess greater thermodynamic stability than the high-affinity forms (‘c–d’), the equilibrium results in what is macroscopically measured as a large population of receptors displaying a low-affinity binding mode. On the other hand, receptors that assume the high-affinity forms (‘c–d’) are responsible for what appears macroscopically as a small population of high-affinity receptors.

Although contact formation between the extracellular domains in the ligand-free state (forms ‘c–d’ in Fig. 1A) induces high-affinity binding, these forms do not necessarily resemble the ligand-bound dimers (forms ‘e–f’ in Fig. 1A) [56]. Binding of the growth factor ligand stabilizes an active extracellular domain dimer (depicted by exposure of the dimerization arm in forms ‘e–f’ in Fig. 1). This in turn stabilizes the active conformation of the TM helical pair that promotes stable formation of the active intracellular dimer. The implicit assumption here is that the JM domain, connecting the TM helix to the kinase domain, is rigid, as predicted by McLaughlin et al. [36]. Because the intracellular dimer is asymmetric, it could presumably be composed of two possible monomeric orientations (forms ‘e–f’ in Fig. 1). Overall, the equilibrium is strongly shifted toward the catalytically active state.

4. Are all ErbBs regulated in the same way?

The ErbB family is ubiquitously distributed throughout the animal kingdom. There are four family members in vertebrates, whereas invertebrates have only one. The first gene duplication, in the early divergence of the vertebrates, generated the ErbB1/ErbB2 and ErbB3/ErbB4 precursors, each of which underwent a second gene duplication event to generate the four receptors present in vertebrates [1]. The unique sequence of each of these four isoforms dictates their particular regulation and signaling. Some examples are described below. The presence of only one ErbB isoform in invertebrates implies that most aspects of its regulatory mechanisms differ from those in vertebrates [1]. In this review we focus on the evolutionary conservation of ErbBs in species that diverged after the generation of four isoforms.

4.1. Regulation of the ligand-less ErbB2 via its extracellular domain

ErbB2 is unique among the ErbBs in that it lacks the layer of inhibitory regulation that is provided by the extracellular domain, which imposes ligand-induced activation. Ligand-free ErbB2 assumes the extended, otherwise ligand-bound conformation of the EGFR [69,70]. Accordingly, the extracellular domain of ErbB2 exhibits sequence variations in residues that participate in inter-subdomain interactions stabilizing the tethered conformation and which are conserved in ErbB-1, -3 and -4. Moreover, ErbB2 undergoes additional inter-subdomain interactions that mimic ligand-mediated contacts in the other ErbBs. Correspondingly, conserved residues that participate in ligand binding in other ErbBs are replaced in ErbB2 by residues that can participate in the inter-subdomain interactions [71]. Regardless of the constantly extended conformation of its

extracellular domain, ErbB2 does not show a tendency to form active homodimers [80]. This might be due to electrostatic repulsion by negative charges on the predicted interface [71]. Nevertheless, ErbB2 efficiently forms heterodimers with other ErbBs [8]. Like ErbB2, ErbB3 is also reluctant to form homodimers, even in response to its ligand, neuregulin [81]. Nevertheless, neuregulin readily induces the formation of the most prevalent and highly efficient ErbB3−ErbB2 heterodimers [8].

4.2. Regulation of the kinase-dead ErbB3 via its TM domain

As mentioned above, the TM segments of most ErbBs display two dimerization motifs and it was suggested that they correspond to the active and inactive conformations [11]. Interestingly, the kinase-dead ErbB3 contains only the dimerization motif that corresponds to the active state according to this suggestion [11]. Thus, ErbB heterodimers that contain ErbB3 have probably lost the layer of negative regulation imposed by the TM domains and hence more readily favor the active conformation. The indications that ErbB2 and ErbB3 lost negative regulation in their extracellular and TM domains, respectively, could explain the observation that ErbB2−ErbB3 heterodimers are extremely potent [8,82].

4.3. Regulation by the intracellular domain

4.3.1. Loss of intramolecular regulation in ErbB3

The monomeric inherent inactive conformation of the kinase domain is stabilized by packing of the activation loop against the αC-helix and its preceding loop [34], for example via contacts between residues Ile735 and Leu837 and between Met742 and Leu834 (Fig. 4B). It is interesting to note that these residues, which are putatively important for the packing, show some variation in ErbB3 (Fig. 4C), whereas they are conserved among other ErbBs. Mutations in these positions have also been found to correlate with malignancy, presumably because of destabilization of the active state; this is discussed further in Section 5.1.1. Overall, our structural and sequence analyses suggest that there are fewer constraints on stabilization of the inherent inactive conformation of the kinase domain of ErbB3 than of other ErbBs. This observation is consistent with the reported lack of intramolecular activity in the kinase-dead ErbB3 [8]. Nevertheless, we have to take into account the activation of another ErbB member by heterodimerization with ErbB3. Accordingly, as described below, the regulation imposed by intermolecular interactions (e.g., via formation of an inactive intracellular dimer) is still maintained in ErbB3.

4.3.2. Negative regulation of all ErbBs by an inactive intracellular dimer

The symmetric and presumably inactive crystallographic EGFR dimer (Fig. 3) shows charge complementarity between the kinase and the C-terminal domains at the interfaces, as described in Section 2.2.1. The positively charged residues on the interfaces in the kinase domain are highly conserved in all four ErbBs. Accordingly, our previous analyses, based on predicted structures, demonstrated that all of the human ErbBs display positively charged patches at the corresponding regions [12]. Evolutionary conservation analyses and electrostatic

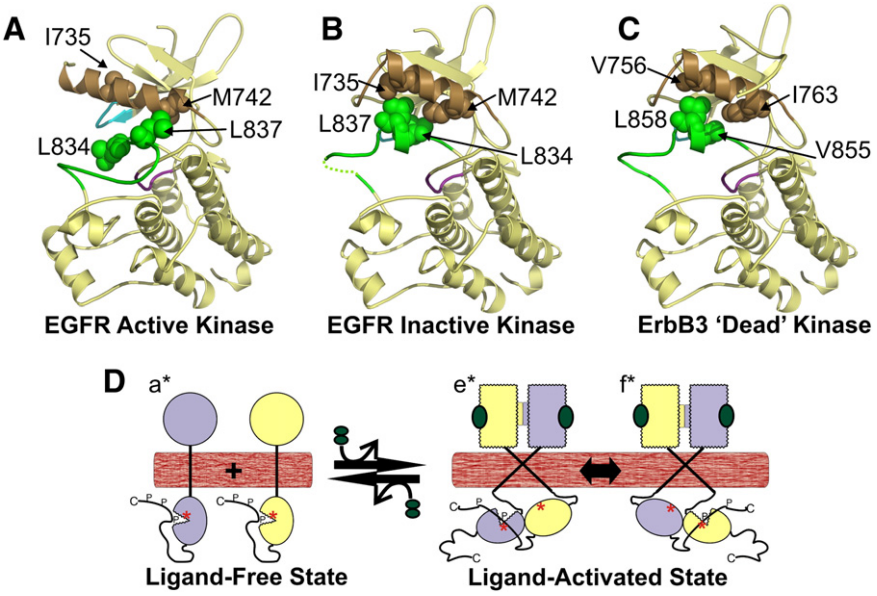


Fig. 4. Destabilizing mutations in regulatory regions. The kinase domain of the EGFR (residues 685−957) is depicted in a yellow ribbon representation. Regulatory elements are depicted by different colors: the activation loop (residues 831−852) in green, the αC-helix (residues 729−745) in brown, the phosphate-binding loop (residues 695−700) in cyan, and the catalytic loop (residues 812−818) in purple. A disordered region within the activation loop is depicted by a dashed curve. Residues shown by mutagenesis to be crucial for stabilizing the inherent inactive conformation of the kinase domain of the EGFR are depicted as space-filled atoms. (A) Active kinase domain of the EGFR (PDB entry — 2gs6 [34]). (B) Inactive kinase domain of the EGFR (PDB entry — 1gs7 [34]). (C) Model of the catalytically inactive kinase domain of ErbB3. (D) Scheme illustrating alterations of the regulatory model (shown in Fig. 1A) by mutations, e.g., in residues Ile735, Met742, Leu834, and Leu837, which are shown in panels A and B. According to the model, the mutations (indicated by red stars) would cause destabilization of the inherent inactive conformation, leading to the formation of an active kinase domain whose phosphorylation is independent of the ligand or intermolecular contacts (form a*). Binding of ligand induces formation of the active dimer (forms e*−f*) that can potentially phosphorylate in *trans* and hence further increase the rate of auto-phosphorylation.

calculations show that those interfaces are not common to other tyrosine kinases [12]. In addition, the C-terminal fragment in contact with the kinase domain in the crystal structure in all ErbBs contains mostly negatively charged residues, even though the entire C-terminal domain is not generally conserved [1]. This implies that the electrostatic complementarity between the kinase domain and the C-terminal fragment might be important for controlling the activity of homo- and heterodimers in the entire ErbB family.

4.3.3. Functional and structural asymmetry of the active state

The active state of the intracellular domain is characterized by formation of an asymmetric dimer of the kinase domains, in which one monomer activates the other [34]. It was previously shown that ErbB heterodimers could phosphorylate in *trans*, i.e., that the active monomer could phosphorylate its neighbor within the dimer, as for example in the apparent phosphorylation of the kinase-dead ErbB3 by its partner [3]. Moreover, the phosphorylation pattern of an ErbB member, and thus the subsequent signal transduction pathways, is dependent on its dimerization partner [83]. Phosphorylation of both kinases would necessitate a switch between the orientations of the two subunits (illustrated by the transition between forms 'e' and 'f' in Fig. 1A). The structural asymmetry implies that at any given time only one of the kinases is catalytically active (represented by the purple kinase in form 'e' in Fig. 1) and can phosphorylate the C-terminal domain of its partner, namely the activating monomer (represented by the yellow kinase in form 'e' in Fig. 1). Interestingly, some ErbB heterodimers show preference for the formation of a specific orientation within the asymmetric dimer, as discussed further below.

4.3.3.1. The kinase-dead ErbB3 favors the role of activating monomer. In heterodimers that include ErbB3, only one direction of interaction, in which ErbB3 is the activating subunit, is functionally productive. Evolutionary conservation analysis of the ErbBs suggests that the interface of the activating monomer within the asymmetric dimer (yellow kinase in form 'e' in Fig. 1) is conserved among all four ErbBs. In contrast, most residues comprising the interface of the activated monomer (purple kinase in form 'e' in Fig. 1) are conserved only in ErbB-1, -2, and -4, while ErbB3 shows sequence variations. Presumably, therefore, an interface in which ErbB3 assumes the position of the activated subunit is energetically less stable. We note that this analysis, which was based on 36 ErbBs from vertebrates and viruses (see Methods), reproduced the conclusions drawn by Zhang and co-workers [34] that were based on a smaller set of human and mouse ErbB sequences.

4.3.3.2. ErbB2 favors the role of the activating monomer within EGFR-ErbB2 heterodimers. ErbB2, uniquely among ErbBs, possesses an additional layer of regulation, mediated by molecular chaperones (primarily Hsp90) that normally help to stabilize the receptor on the cell surface [80,84]. Hsp90 binds to ErbB2 and restrains signaling by limiting the formation of heterodimers of ErbB2 with other ErbBs, which would require the stripping of Hsp90 [80]. Binding of Hsp90 to ErbB2 is mediated through the loop that follows the αC-helix, and specifically within residues Gly776−Ser783 [80]. This region is located close to the asymmetric active dimer's interface, which is contributed by the N-lobe of the activated monomer (purple kinase in form 'e' in Fig. 1). Accordingly, we hypnotize that binding of Hsp90 might lead to the preferential formation of EGFR−ErbB2 heterodimers in which EGFR is found in the position of the active monomer (purple kinase in form 'e' in Fig. 1) that phosphorylates the C-terminal domain of ErbB2 (yellow kinase in form 'e' in Fig. 1). In accordance with the above hypothesis, some pieces of evidence suggest that within EGFR-ErbB2 heterodimers, a catalytically competent EGFR is required for the signaling of ErbB2, both in normal and in cancerous conditions [85,86]. Furthermore, the signal transduction mediated by ErbB2 is blocked by the EGFR-specific kinase inhibitor gefitinib [87,88].

Taken as a whole, the experimental evidence corresponds to a preferred asymmetric formation of an EGFR−ErbB2 heterodimer, in which the EGFR is activated by ErbB2 and phosphorylates it, leading to cellular signaling that is specific to ErbB2. This asymmetry might be related to the binding of Hsp90 to ErbB2, as mentioned above. It is interesting to note that alteration in the Hsp90-binding loop, as in a certain oncogenic mutant discussed in Section 5.1.4, changes the normal orientation within EGFR−ErbB2 dimers. Overall, the above findings elegantly manifest the asymmetry in the ErbBs' function within heterodimers and its importance for normal signaling. This functional asymmetry can now be clarified by the structural asymmetry of the active state observed by Zhang and co-workers [34].

In the case of heterodimerization between ErbB2 and the kinase-dead ErbB3, ErbB2 must play the role of the activated monomer, which probably requires pre-striping of Hsp90. Indeed, inhibition of Hsp90 potentiates the formation of active ErbB2-ErbB3 heterodimers [80]. Since ErbB2 and ErbB3 lost the negative regulatory layer imposed by the extracellular and TM domains, respectively (see Sections 4.1 and 4.2), their heterodimerization is extremely potent [8] despite the restrains imposed by Hsp90. We note that the binding of Hsp90 to the active dimerization interface apparently provides an additional explanation at the molecular level of the reluctance of ErbB2 to form active homodimers [80], in addition to limitation imposed by the extracellular domain [71] (see Section 4.1).

5. The role of the ErbB family in pathologies

Aberrant activation of the ErbBs has been described mostly in relation to cancer [2,89]. However, activation of the EGFR was found to be related also to kidney lesions, and ErbB4 and its ligand neuregulin-1 are involved in the pathogenesis of schizophrenia. Moreover, both EGFR and ErbB2 bind to and are activated by viruses and bacteria, which is a requirement for the pathogens' biological activity [4]. All of the above ErbB-associated pathological processes require activation of the receptors. Deficiency of ErbBs would be extremely damaging and even lethal to the developing embryo, which is presumably why it is not observed clinically.

5.1. ErbBs and cancer

EGFR is highly expressed in many cases of non-small cell lung cancers (NSCLC) (88–99%) [90], head and neck cancers (80%) [91], and gliomas (40%), as well as in some pancreatic and breast tumors [3,92]. Amplification and over-expression of ErbB2 have been demonstrated in 20–25% of breast cancers and also occur, albeit at lower frequencies, in lung, pancreatic, colon, endometrial and ovarian cancers [3,93,94]. Accordingly, the ErbB family has become an attractive target for anticancer therapy [95].

Regardless of its frequent involvement in cancers, over-expressed EGFR is still dependent on its ligand, EGF, for activation [37,96]; over-expression in itself is not prognostic of survival in NSCLC [90]. Correspondingly, regardless of the high percentage of EGFR involvement in NSCLC, only a small fraction of patients benefit from treatment with specific EGFR kinase inhibitors. Mutations were recently detected in the kinase domain of the EGFR in these patients [97,98] and proved to be oncogenic, i.e., to increase the receptor's basal kinase activity [37,90,96,99,100]. We suggest that these findings are related to the fact that random contact formation, induced by over-expression, is not by itself sufficient to provoke activation, although, statistically speaking, it probably increases the amount of active receptors within the cell. More prominent effects on activation are probably displayed by mutations that have the potential to alter the inherent activity of the receptor or to shift the dynamic equilibrium in Fig. 1 toward the active state.

Numerous mutations in ErbB family members have been detected in tumor tissues. For example, mutations in EGFR were found to occur in approximately 20% of lung cancers [101]. Mutations in ErbB2 have been reported in lung cancers, although at lower rates than EGFR mutations (1−4%) [93,102–108], as well as in gastric, colorectal and breast cancers [105]. The mutations found in the EGFR and ErbB2 appear to be mutually exclusive, as they have never been found together in individual tumors or cell lines [101,102,109]. Interestingly, most of the alterations found in ErbB2 overlap with the analogous structural regions of those found in the EGFR [93], suggesting that they are functional [102]. Correspondingly, some of the prevalent mutations in EGFR and ErbB2 have been experimentally examined and found to be oncogenic (these mutations are listed in the supplementary information as Table 1S). However, the functional effect of rarer alterations (listed in Table 2S in the Supplementary information) is less clear [102]. Recently, mutations in ErbB4 were also detected in NSCLC, as well as in gastric, colorectal and breast carcinomas, although at lower frequencies (1−3%) than mutations in the EGFR [108].

In the following sections we use the regulation model shown in Fig. 1A to suggest molecular interpretations, based on structural and evolutionary considerations, of the effect of known cancer-causing mutations. We use the same framework to predict the possible effects of other ErbB mutations found in cancer cells that have not been characterized experimentally (listed in Table 2S).

5.1.1. Missense mutations in the regulatory activation loop

In the inactive state of the EGFR, the activation loop (residues 831−852) is packed against the αC-helix (residues 729−745) and its preceding loop (residues 723−728) such that the elements needed for catalysis are misplaced (Fig. 2). Destabilization of the packing will cause a shift toward the activated state of the kinase domain. Such an effect was indeed predicted for the oncogenic mutations L834R, which represents 41−43% of the mutations in lung cancers, and for L837Q (L858R and L861Q in pre-mature EGFR numbering, respectively) [101,109]. L834 and L837 are located on the activation loop and participate in interactions with hydrophobic residues in the αC-helix in the inactive conformation (Fig. 4B), while in the active conformation they are quite exposed to the solvent (Fig. 4A). Correspondingly, mutations in these hydrophobic residues with polar/charged ones lead to a ligand-independent activation [37,90,96,99,100], via destabilization of the inactive monomeric state [34]. In accordance with clinical findings, both EGFR mutants are more sensitive than the wt to inhibition by specific kinase inhibitors, which preferentially bind to the active conformation of the kinase domain [90,96–100]. This further suggests that the mutants preferentially assume the active conformation. Furthermore, neither L834R nor L837Q appears to impair the maximal response to EGF [37], implying that the conformation of the mutants' kinase domain could be fully competent for catalysis.

The ligand-free state of the EGFR is characterized by interconversion between several conformations, each displaying a different binding affinity for the growth factors (Fig. 1A). Because, according to the model, the lower affinity conformations are energetically more stable than the higher affinity conformations, macroscopic measurements of binding affinities point to large and small populations of low- and high-affinity receptors, respectively, as discussed in Section 2.5. We noted above that alterations in EGFR can potentially affect the overall activity and ligand-binding affinity of the receptor by interference with the dynamic equilibrium depicted in Fig. 1. Mutations such as L834R and L837Q, which destabilize the inactive monomeric conformation of the kinase domain, are expected to induce monomers having ligand-independent, catalytically competent kinase domains that are sufficient to transduce signals (form a* in Fig. 4D). In addition, the L834R mutation displays approximately twofold higher affinity for EGF in what was macroscopically measured as the low-affinity binding mode [37]. The effect is rather small, but microscopically, this mutation may shift the dynamic equilibrium in the ligand-free state toward higher affinity forms. Nevertheless, the EGFR mutants could not entirely mimic the ligand-bound active state because binding of the ligand further increased auto-phosphorylation of the receptor [37]. This latter event may be related to further stabilization of active dimers by the binding of the ligand; these dimers could phosphorylate in *trans* (forms 'e−f' in Figs. 1A and 4D).

Interestingly, similarly to the EGFR, other ErbB members as well as other kinases also displayed substitutions in the positions corresponding to Leu834 and Leu837 (Table 1S). Overall, the high frequency of mutations in these two positions points to their crucial importance for regulation. A reasonable assumption is

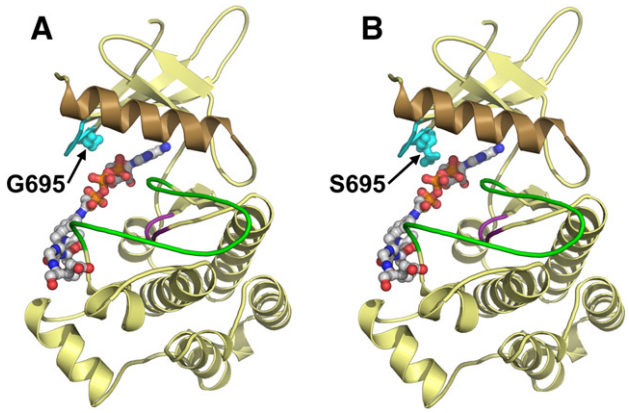


Fig. 5. Mutations in the phosphate-binding loop. The kinase domain of the EGFR (residues 685−957) is depicted in a yellow ribbon representation. Regulatory elements are depicted by different colors: the activation loop (residues 831−852) in green, the αC-helix (residues 729−745) in brown, the phosphate-binding loop (residues 695−700) in cyan, and the catalytic loop (residues 812−818) in purple. Position 695 is displayed in a balls-and-sticks representation. (A) The wt EGFR (PDB entry — 2gs6 [34]). (B) Model of the G695S mutant found in cancer cells.

that EGFR alterations that destabilize the inherent inactive state of the kinase domain are likely to be oncogenic. Correspondingly, it is interesting to note that mutations in additional residues that contribute to packing of the activation loop in the inactive state, for example I735A and M742A located on the αC-helix (Fig. 4B), show robust elevated basal activity *in vitro*, although they were not detectable in tumor cells [37].

The importance of the mutation-bearing residues is reflected also in the pattern of their substitution during evolution of the ErbB family. Leu837 is conserved as a hydrophobic residue, mostly leucine. However, similarly to the L837Q mutant found in human tumors, two oncogenic viral variants of the EGFR also display replacement by glutamine in this position, further pointing to a correlation between mutations in this residue and malignancy. Its neighbor, Leu834, is totally conserved in all catalytically active ErbBs, indicative of its important role. However, this position is substituted to valine or isoleucine in the kinase-dead ErbB3 (Val855 in human ErbB3; Fig. 4C). Similarly, the positions corresponding to Ile735 and Met742, which display other activating mutations mentioned above, are unique to ErbB3. Specifically, Met742 is conserved as methionine in all ErbBs, except for ErbB3 in some species that contain isoleucine or valine (Ile763 in human ErbB3; Fig. 4C). In addition, whereas the catalytically active ErbBs display large hydrophobic residues (such as phenylalanine, methionine, leucine, and isoleucine) in the position corresponding to Ile735, this position in the kinase-dead ErbB3 is occupied by valine, a smaller residue (Val756 in human ErbB3; Fig. 4C). It is interesting to note that this position is also occupied by valine in two oncogenic variants of EGFR found in spiketail (*Xiphophorus xiphidium*) and southern platyfish (*Xiphophorus maculates*), relating this substitution to the unstable inactive state. Overall, ErbB3 displays fewer hydrophobic contacts between the αC-helix and the activation loop than the other ErbBs. For example, the contacts between Ile735 and Leu837 as well as between Met742 and Leu834 within the EGFR (Fig. 4B) are missing in ErbB3 (Fig. 4C). Together, these findings lead us to suggest that the inactive conformation of the kinase domain of ErbB3 is less stable than in the other ErbBs isoforms, and that it might even assume a constantly active conformation. Apparently, there was no evolutionary constraint to stabilize the inactive state of ErbB3, as opposed to other ErbBs. This bears a crucial consequence on the regulation mechanism of the ErbBs, which is apparently far more promiscuous in ErbB3.

5.1.2. Mutations in the phosphate-binding (P)-loop

G695S (G719S in the pre-mature EGFR numbering) is another mutation that displays heightened basal activity and is found in cancer patients [37,90,96,100]. Gly695 is the first glycine in the G-X-G-X-X-G motif in the P-loop of the kinase domain, which determines the position of ATP during catalysis (Fig. 5) and accordingly is conserved in the ErbBs as well as in other tyrosine kinases. Nevertheless, the G695S mutation does not impair the maximal response to EGF and thus allows the proper positioning of the catalytic elements to be preserved [37]. The P-loop does not display noticeable conformational changes between the active and inactive conformations of the kinase domain. Thus, in contrast to residues in the activation loop and the αC-helix, Gly695 does not appear to participate in stabilizing the inactive conformation. The mutation might exert an effect by directly influencing the phosphate transfer reaction via lowering of the dissociation rate of ATP (as well as that of ATP-analog inhibitors). Accordingly, G695S is more sensitive than the wt EGFR to ATP-analog EGFR kinase inhibitors [96,100]. However, because the inhibitors display higher affinity for the active conformation, G695S should be less sensitive to the inhibitors than the L834R and L837Q mutations that induce a ligand-independent active form. Overall, we expect the G695S mutation to increase the catalytic efficiency, but not to interfere with the dynamic equilibrium in the model of Fig. 1. Experimental results indeed showed that G695S does not alter the binding affinity for the ligand [37].

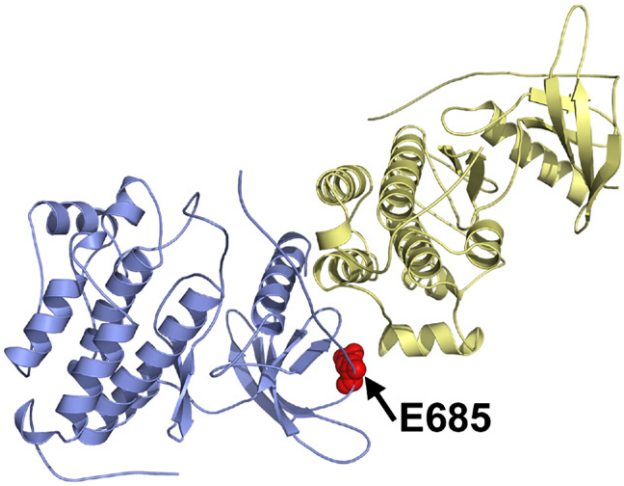


Fig. 6. Mutation at the interface of the intracellular active dimer. The asymmetric active dimer of the kinase domain (colored yellow and purple) of the EGFR (residues 685−967; PDB entry — 1m17 [32]) is depicted in a ribbon representation. Glu685, located on the activated monomer (in light purple), is depicted as a red sphere. This residue is substituted in some cancer cells.

5.1.3. Mutations at the interface of the active asymmetric dimer

Substitutions of alanine or glycine for Glu685 (709 in the pre-mature EGFR numbering) have been reported in lung cancers [110,111]. Like the other missense mutations, E685G also showed increased basal auto-phosphorylation and a heightened sensitivity to EGFR kinase inhibitors such as gefitinib [90]. Interestingly, Glu685 is located on the N-lobe of the EGFR as part of the interface on the activated monomer within the asymmetric dimer (purple kinase in Fig. 6). According to the model, this interface is important for the activation of the catalytically active ErbBs, but is irrelevant for the kinase-dead ErbB3 [34], as discussed in Section 4.3.3. Accordingly, Glu685 is conserved within the catalytically active ErbBs, but not in some species of ErbB3. We suggest that the E685G mutation might further stabilize the formation of the active dimer by shifting the dynamic equilibrium of EGFR towards the active forms (e.g., form 'c' in Fig. 1A), leading to an increase in basal activity. Another possibility is that within heterodimers comprising EGFR, mutations at the interface might change the native orientation of the monomers by altering the roles of the activating and the activated monomers (see Section 4). The resulting modification in signal transduction could also lead to oncogenic transformation (see Section 5.1.4 for an example).

5.1.4. Mutations in the αC-helix and its surrounding regions

5.1.4.1. EGFR exon19 deletions in the N-terminus of the αC-helix and its preceding loop. In addition to the missense mutations discussed above, other oncogenic variations in the EGFR correspond to different deletions within residues 722−735 in *exon19*. As with the former mutations, when tested *in vitro* such deletions show increased basal activity compared to wt EGFR [37,90,96,99], as well as higher sensitivity to EGFR kinase inhibitors [90,96,97,99]. The region displaying the deletions constitutes the loop preceding the αC-helix and its N-terminus, which participate in interactions with the activation loop in the inactive conformation (Fig. 7A). The crystal structure of the inactive conformation of the kinase domain [34] indeed displays pronounced rigidity in this region, in contrast to the marked flexibility observed in the active conformation [32,34]. Thus, such deletions are predicted to lead to destabilization of the inactive conformation of the kinase domain and to an increase in basal activity, similarly to the L834R and L837Q mutations discussed in Section 5.1.1.

We note that although the deletions destabilize inhibitory interactions, the maximal catalytic activity was diminished [37], possibly because the αC-helix is involved in mediating the active intracellular dimer (Fig. 7C). Accordingly, the ΔL723-P729insS mutant (a 723−729 deletion together with insertion of a serine residue) was shown to abolish the high-affinity binding mode and reduce the low-affinity binding mode by twofold [37] (see Section 2.5 for discussion of ligand-binding affinity). This corresponds to destabilization of the active intracellular dimer constituting the high-affinity forms (forms 'c−d' in Fig. 1A), and results in a general shift toward lower affinity states.

5.1.4.2. Insertions of exon20 in the loop following the αC-helix. *Exon20* insertions have been detected both in the EGFR and in ErbB2, and were shown to be activating alterations. They are discussed in the following sections.

5.1.4.3. Exon20 insertions within the EGFR destabilize the inactive dimer. In contrast to the EGFR alterations discussed above, the *exon20* insertion mutations have not been reported in the group of clinical responders to EGFR kinase inhibitors. Nevertheless, they were detected in few recent large-scale studies [90,107,110,111] and reportedly account for 3.7−5% of the mutations in lung cancers [101,109]. The insertions were detected in the C-terminus of the αC-helix and the region following it, within residues 744−749. One such insertion, D746insNPG, i.e., insertion of residues Asn-Pro-Gly following Asp746 (Asp770 in pre-mature EGFR numbering), was tested *in vitro* and showed heightened basal activity [96]. In contrast to the previously discussed mutations and in accordance with clinical findings, this insertion mutant was remarkably insensitive to the EGFR kinase inhibitors gefitinib and erlotinib; it

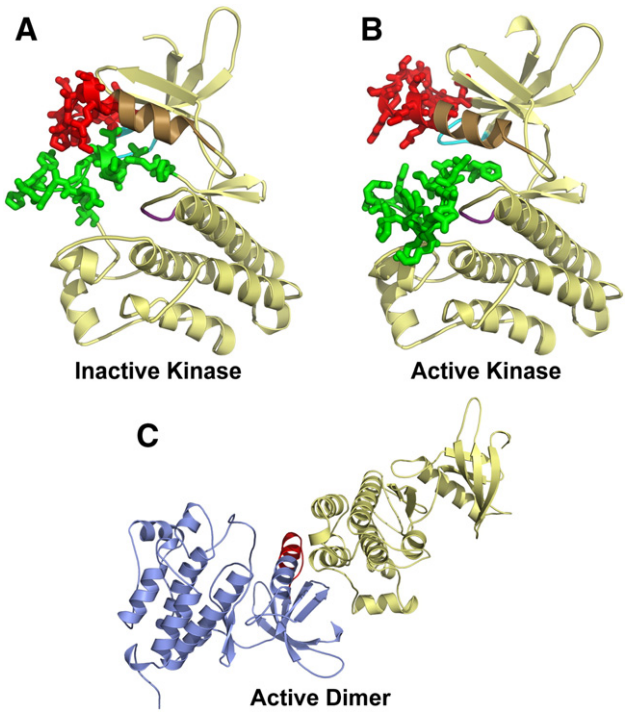


Fig. 7. Deletions in *exon19* in the EGFR. (A−B) The kinase domain of the EGFR (residues 685−957), in the inactive (A) (PDB entry — 1gs7 [34]) and active (B) (PDB entry — 2gs6 [34]) conformations, is depicted in a yellow ribbon representation. Regulatory elements are depicted by different colors: the activation loop (residues 831−852) in green, the C-terminus of the αC-helix (residues 736−745) in brown, the phosphate-binding loop (residues 695−700) in cyan, and the catalytic loop (residues 812−818) in purple. The region showing deletions in cancer cells (residues 722−735; displayed in a balls-and-sticks representation) is colored red. Residues comprising the activation loop are also displayed in a balls-and-sticks representation. Interactions between the region showing deletions in cancer cells (red) and the activation loop (green) in the inactive state (A) are noticeable. (C) The asymmetric dimer of the kinase domains (the monomers are colored yellow and light purple) of the EGFR (residues 685−957; PDB entry — 1m17 [32]) is depicted in a ribbon representation. The region showing deletion in cancer cells (residues 722−735) is colored red in the activated (purple) monomer.

was more sensitive, however, to treatment with the irreversible inhibitor CL-387,785 [96], which is covalently bound to EGFR in residue C773 [112].

The region following the αC-helix forms an exposed loop on the kinase domain. This loop displays similar orientations in the active and inactive states and is not predicted to play a role in stabilizing the monomeric inactive state. Nevertheless, it is located close to the interface of the symmetric, putatively inactive, crystallographic dimer of the kinase domain (see Section 2.2.1), facing the equivalent loop from the second monomer (Fig. 8). Prediction of the structure of the D746insNPG alteration showed clashes between insertions within the symmetric dimer (Fig. 8), possibly leading to destabilization of the inactive form (form 'b' in Fig. 1), and prompting activation by increasing the fraction of receptors in the active form. The mechanism leading to insensitivity to the inhibitors is not yet clear.

5.1.4.4. Exon20 insertions in ErbB2 alter orientation within EGFR–ErbB2 heterodimers. The most prevalent ErbB2 alterations found to date in cancer cells are insertions in *exon20*. These insertions were found within residues 774–783 of ErbB2, corresponding to residues 742–751 of the EGFR [91,93,102–104,107,113]. The most common of these alterations, G776insYVMA, was recently shown to exhibit a more potent auto-catalytic activity than wt ErbB2 [88]. Interestingly, this insertion is located within the binding site of Hsp90 [80].

In Section 4.3 we discussed functional asymmetry within EGFR–ErbB2 heterodimers, wherein ErbB2 is preferentially the activating monomer and makes contact with the EGFR via its C-lobe (the yellow kinase in form 'e' in Fig. 1). The EGFR in turn takes on the role of activated monomer and phosphorylates ErbB2, leading to specific signal transduction. This orientation may be imposed by the binding of Hsp90 to the dimerization interface on the N-lobe of ErbB2 [80] (see Section 4.3.2). According to the model, alterations in the Hsp90 binding loop, as in the G776insYVMA mutant, would prevent the binding of Hsp90 and enable ErbB2 to interact with the EGFR via its N-lobe. As a result, ErbB2 preferentially assumes the role of the activated monomer (the purple kinase in form 'e' in Fig. 1). Correspondingly, experiments have shown that in contrast to wt ErbB2, the G776insYVMA mutant phosphorylates its heterodimeric partner EGFR in a manner that does not require a catalytically active EGFR. Accordingly, in contrast to the situation in wt dimers, this activation was blocked by direct ErbB2 inhibitors such as trastuzumab, lapatinib, and CI-1033, but not by the EGFR-specific kinase inhibitors erlotinib or gefitinib [88]. In addition, binding of Hsp90 restrains cellular signaling by ErbB2 [80], indicating that the kinase activity of the insertion mutant is more potent than that of the wt [88].

We note that an additional contribution prompting formation of the active dimer by the mutation might be related to destabilization of the inactive dimer, as in the case of *exon20* insertions within the EGFR. Interestingly, heterodimers constituting the EGFR and the G776insYVMA ErbB2 mutant do not respond to ligand binding, and do not require contact formation between their extracellular domains [88]. A possible explanation is that the association of ErbB2 via its N-lobe to the EGFR's C-lobe is energetically stable and that, together with a probable loss of control by the inactive dimer, it could preferentially occur via random contacts. Under normal conditions, this interaction is prevented mainly by binding of Hsp90 to the dimerization interface and the formation of an inactive dimer.

5.1.4.5. The S744I mutation at the C-terminus of the αC-helix. Another residue that undergoes substitution in tumor cells is Ser744, located at the C-terminal end of the αC-helix. Compared to the wt, the S744I mutant displays heightened basal kinase activity and greater sensitivity to EGFR kinase inhibitors such as gefitinib [90]. In contrast to mutations in the activation loop and the αC-helix, which are buried inside the hydrophobic core and thereby stabilize the inactive conformation, Ser744 is relatively exposed to the solvent. The effect, therefore, is presumably not related to destabilization of the inherent inactive conformation.

Ser744 is conserved in ErbBs except for ErbB2, in which a glycine residue occupies this position (Gly776 in human ErbB2). This region in ErbB2 indeed displays the unique ability, found in the ErbBs, to bind Hsp90 [80]. Interestingly, an equivalent G776S mutation in ErbB2 was found in gastric tumors [93]. The equivalent locations of the EGFR-S774G and ErbB2-G776S mutations might point to a common molecular effect. Because this position is close to the interfaces of the active

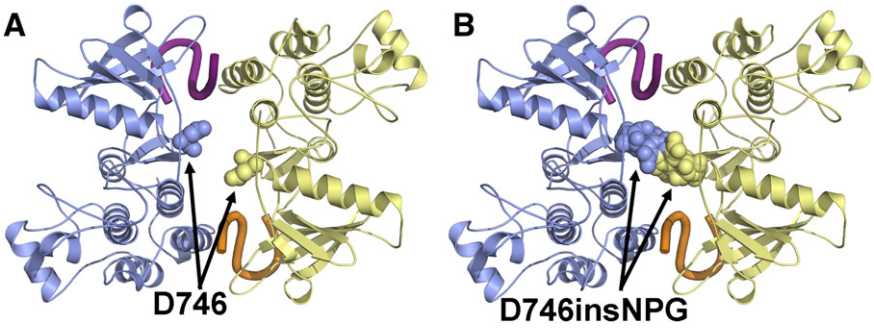


Fig. 8. Insertions in *exon20* in the EGFR. The intracellular symmetric inactive dimer (PDB entry — 1m17 [32]) is depicted in a ribbon representation. The two kinase domains are colored yellow and light purple and their following C-terminal fragments are depicted as orange and purple tubes, respectively. (A) wt EGFR; Asp746 is depicted in space-filled atoms. (B) Model of the EGFR D746insNPG mutant. Residues comprising this insertion are depicted as space-filled atoms. Clashes within the inactive dimer, resulting from the insertion, are noticeable.

dimer, the mutation might contribute to alteration of the native orientation within active asymmetric heterodimers, as in the ErbB2 *exon20* insertions discussed above.

5.1.5. Mutations found in tumor cells that have not been yet analyzed in vitro

A large set of mutations has been found to exist in tumor cells. Those occurring frequently have been analyzed *in vitro* for their effects on kinase activity, as discussed in Sections 5.1.1–5.1.4. The oncogenic nature of other mutations of the EGFR, ErbB2, and ErbB4 has not yet been established. Based on the structural locations of these residues and their evolutionary conservation patterns within the ErbB family, we offer (Table 2S) a prediction as to the nature of their substitutions, i.e., whether it is likely to be damaging or neutral. Some examples are provided below.

5.1.5.1. The R752C mutation in the EGFR is predicted to be damaging. R752C (residue 776 in the pre-mature EGFR numbering) was found as a second mutation to L834R in patients with lung tumors sensitive to the EGFR kinase inhibitor erlotinib [98]. Arg752 is conserved in ErbBs and is located at the interface of the symmetric, putatively inactive crystallographic dimer (Fig. 3). This residue, which is connected by a salt bridge to Asp990 located on the C-terminal domain, plays a role in the polar network stabilizing the dimer (see Section 2.2.1). Thus, this mutation is predicted to lead to destabilization of the inactive dimeric state. Interestingly, a mutation in the corresponding residue of ErbB4 (R782Q) [108] was also reported in cancer cells, supporting our assumption that these mutations are indeed oncogenic.

5.1.5.2. The L723F mutation in the EGFR is predicted to be damaging. The L723F mutation (residue 747 in the pre-mature EGFR numbering) was observed in lung cancers [114]. Residue Leu723 is located in the loop preceding the αC-helix, at the beginning of the *exon19* deletions found in cancer patients, and is in contact with the activation loop in the inactive state, i.e., with residues Leu834, Leu837, and Leu838 (Fig. 9). Leu723 is conserved in all the ErbBs except the kinase-dead ErbB3, in which this position is occupied by isoleucine, and which probably exhibits a constant active conformation of the kinase domain (see Section 5.1.1). In view of its conservation pattern and structural location, we predict that the Leu723 mutation might participate in destabilizing the inactive state of the kinase domain, similarly to the *exon19* deletions and other missense mutations in the αC-helix and activation loop. Interestingly, the corresponding residue in ErbB2 (Leu755) also displays substitutions in cancer cells [93,104,107], suggesting that these mutations are indeed oncogenic.

5.1.5.3. The N857S mutation in ErbB2 is predicted to be neutral. The ErbB2 N857S mutant (corresponding to Gln825 in EGFR) was reported in an ovarian tumor [93]. A model of ErbB2 predicted that Asn857 is located in a loop on the back side of the catalytic site and is exposed to the solvent. This position shows evolutionary variation in ErbBs. Based on analyses of evolutionary conservation and structural location, we predict that substitution of the mutation N857S is neutral in its effect.

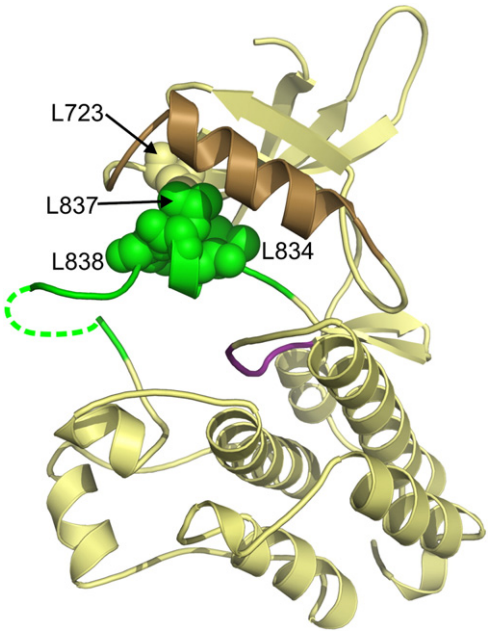


Fig. 9. A predicted oncogenic mutation. The kinase domain of the EGFR (residues 685−957) in the inactive conformation (PDB entry — 1gs7 [34]) is depicted in a yellow ribbon representation. Regulatory elements are depicted by different colors: the activation loop (residues 831−852) in green, the αC-helix (residues 729−745) in brown, and the catalytic loop (residues 812−818) in purple. The disordered region in the crystal structures is depicted by a dashed line. Leu723, which was found to be mutated in cancer cells, is depicted in space-filled atoms. In this inactive conformation Leu723 interacts with Leu834, Leu837, and Leu838 on the activation loop (depicted in green space-filled atoms). We predict that the L723F substitution leads to destabilization of the inactive conformation.

6. Methods

6.1. Evolutionary conservation analysis of the ErbB family

Sequences of the kinase domain of ErbB isoforms from various species were collected from the UNIPROT database [115] and the NR database from NCBI, using PSI-BLAST [116]. The resulting 306 sequences were aligned using MUSCLE [117]. Fragmented or redundant sequences were removed from the alignment. In addition, sequence variants and mutants were discarded, along with proteins sequenced by a whole-genome shotgun project, because they are viewed as preliminary data. The resulting multiple sequence alignment (MSA) contained 48 sequences of ErbBs from vertebrates and invertebrates. The invertebrate sequences were removed, and an MSA of 36 ErbBs from vertebrates (and viruses) was used to calculated evolutionary conservation scores using a Bayesian method [118] as implemented in the ConSurf web-server (http://consurf.tau.ac.il/) [119].

6.2. Structure prediction and analyses

The structures of the kinase domains of ErbB2, ErbB3, and mutants found in cancer cells were modeled on the basis of the structure of the kinase domain of the EGFR (PDB entry — 1m17 [32]) using the NEST program [120] with default parameters. The solvent-accessible area was calculated using the SURFV program with a probe sphere of radius 1.4 Å and default parameters [121].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbcan.2007.08.001.

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