Breakthroughs and Views

# The ErbB/HER receptor protein-tyrosine kinases and cancer[☆]

Robert Roskoski Jr.[*]

*Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, 1100 Florida Avenue,
New Orleans, LA 70119, USA*

## Abstract

The ErbB/HER protein-tyrosine kinases, which include the epidermal growth factor receptor, consist of a growth-factor-binding ectodomain, a single transmembrane segment, an intracellular protein-tyrosine kinase catalytic domain, and a tyrosine-containing cytoplasmic tail. The genes for the four members of this family, ErbB1–ErbB4, are found on different human chromosomes. Null mutations of any of the ErbB family members result in embryonic lethality. ErbB1 and ErbB2 are overexpressed in a wide variety of tumors including breast, colorectal, ovarian, and non-small cell lung cancers. The structures of the ectodomains of the ErbB receptors in their active and inactive conformation have shed light on the mechanism of receptor activation. The extracellular component of the ErbB proteins consists of domains I–IV. The activating growth factor, which binds to domains I and III, selects and stabilizes a conformation that allows a dimerization arm to extend from domain II to interact with an ErbB dimer partner. As a result of dimerization, protein kinase activation, *trans*-autophosphorylation, and initiation of signaling occur. The conversion of the inactive to active receptor involves a major rotation of the ectodomain. The ErbB receptors are targets for anticancer drugs. Two strategies for blocking the action of these proteins include antibodies directed against the ectodomain and drugs that inhibit protein-tyrosine kinase activity. A reversible ATP competitive inhibitor of ErbB1 (ZD1839, or Iressa) and an ErbB1 ectodomain directed antibody (IMC-C225, or Erbitux) have been approved for the treatment of non-small cell lung cancer and colorectal cancer, respectively. An ErbB2/HER2 ectodomain directed antibody (trastuzumab, or Herceptin) has also been approved for the treatment of breast cancer. Current research promises to produce additional agents based upon these approaches.
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The ErbB/HER protein kinases, which include the epidermal growth factor receptor, are among the most studied cell signaling families in biology [1]. This line of investigation was initiated by Stanley Cohen who first described EGF, its receptor, and its biochemical actions [2]. The X-ray crystallographic structures of the ectodomain of ErbB family members interacting with EGF, TGF-α, and a therapeutic monoclonal antibody have shed light on the unique biochemical and biophysical properties of these proteins [3]. Moreover, the structure of the protein kinase domain of the EGF receptor has also been determined [4].

Protein kinases are enzymes that play a key regulatory role in nearly every aspect of cell biology [5]. They regulate apoptosis, cell cycle progression, cytoskeletal rearrangement, differentiation, development, the immune response, nervous system function, and transcription. Dysregulation of protein kinases occurs in a variety of diseases including cancer, diabetes, and autoimmune, cardiovascular, inflammatory, and nervous disorders. Considerable effort has been expended to determine the physiological and pathological functions of protein-kinase signal transduction pathways during the past 30 years.

Protein kinases catalyze the following reaction:

$$MgATP^{1-} + protein\text{-}OH \rightarrow Protein\text{-}OPO_3^{2+} + MgADP + H^+$$

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[*] Corresponding author. Fax: 1-504-619-8775.
*E-mail address:* biocrr@lsuhsc.edu (R. Roskoski Jr.).

Based upon the nature of the phosphorylated –OH group, these enzymes are classified as protein-serine/threonine kinases and protein-tyrosine kinases. Hunter and co-workers [6] identified 478 typical and 40 atypical protein kinase genes in humans (total 518) that correspond to about 2% of all human genes. The family includes 385 serine/threonine kinases, 90 protein-tyrosine kinases, and 43 tyrosine-kinase like proteins. Of the 90 protein-tyrosine kinases, 58 are receptor and 32 are non-receptor kinases. There is a small group of enzymes, which includes MEK, that catalyzes the phosphorylation of both threonine and tyrosine on target proteins. These enzymes, which closely resemble and are classified as serine/threonine kinases, are called dual specificity kinases. The protein kinase family is the largest enzyme family and the fourth largest gene family in humans [7]. Moreover, there are 106 protein kinase pseudogenes.

The ratio of protein phosphoserine/phosphothreonine/phosphotyrosine in normal animal cells is about 3000/300/1 [8]. Despite the paucity of protein-phosphotyrosines, they play key roles in signal transduction. Protein-serine/threonine kinases typically phosphorylate exogenous proteins, and this may be associated with amplification (one protein kinase molecule phosphorylates many substrate molecules). In contrast, protein-tyrosine kinases are not usually associated with amplification. They undergo autophosphorylation and catalyze the phosphorylation of a few exogenous substrate molecules. In growth factor stimulated cells, the most abundant phosphotyrosines occur in the stimulated receptor itself. The resulting protein-tyrosine phosphates serve as docking sites for molecules that transmit downstream signals, which often include the activation of protein-serine/threonine kinases. Amplification accounts for the high ratio of phosphoserine and phosphothreonine to phosphotyrosine in cells.

## Protein-tyrosine kinases and cancer

Many protein kinases are implicated in the mechanisms leading to malignancies [9]. In 1980, Hunter and Sefton [8] demonstrated that the Rous sarcoma virus (v-src) oncogene product is a protein-tyrosine kinase. Later work showed that v-src (the viral protein) and c-src (the cellular homologue) are non-receptor protein-tyrosine kinases.

Cohen and co-workers discovered that the receptor for epidermal growth factor is a protein-tyrosine kinase, the first receptor protein-tyrosine kinase to be described [10–12]. These studies showed that a single integral membrane protein recognizes the growth factor and possesses protein-tyrosine kinase activity. At the time, these results were revolutionary. Later work showed that v-erbB is homologous to the EGF receptor [13]. This established a mechanistic link between growth control and tumorigenesis. Many neoplasms are associated with EGF receptor activation, which can result from mutation of the receptor, its overexpression, or from EGF receptor stimulation through autocrine loops involving excess production of its growth factors [9,14,15].

## The ErbB/HER growth factor ligands

The EGF family of ligands, which consists of about a dozen members, has an EGF-like domain and three disulfide-bonded intramolecular loops [16]. These peptide ligands are expressed in the extracellular domain of transmembrane proteins and are generated by regulated proteolysis to yield growth factors that contain 49–85 amino acids.

The EGR receptor family has four members [3]. The first family member is the EGF receptor [17]; its human gene is designated ErbB1 after the v-erbB oncogene of avian erythroblastosis virus. The other ErbB gene family members and names are provided in Fig. 1. The ErbB receptors function as dimers or higher oligomers [1]. The members of the ErbB family can form four homodimers and six heterodimers for a total of 10 distinct states.
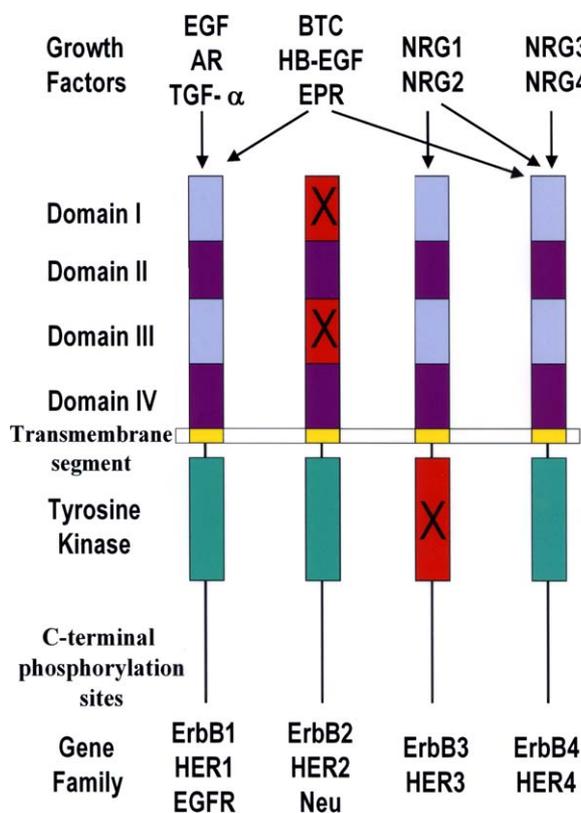


Fig. 1. Epidermal growth factor family of ligands and the ErbB gene family. The topology of the receptor proteins is indicated. The inactive ligand-binding domains of ErbB2 and the inactive kinase domain of ErbB3 are denoted with an X.

Binding of ligands to the ectodomain results in allosteric transitions leading to receptor dimerization, protein kinase activation, *trans*-autophosphorylation, and initiation of signaling cascades [1]. Dimerization, kinase activation, and *trans*-phosphorylation occur in the plasma membrane of cells and in detergent-solubilized purified receptors. Autophosphorylation shows a parabolic dependence on receptor concentration indicating that autophosphorylation occurs in *trans* [18]. Besides activating autophosphorylation at tyrosine residues 992, 1068, 1086, 1148, and 1173, protein kinase C catalyzes the phosphorylation of threonine 669 producing a less active ErbB1 receptor [17].

### The ErbB family of receptor protein-tyrosine kinases

The various ErbB growth factors have different specificities for the receptor family members (Fig. 1). For example, EGF and TGF-α have high affinity for the EGF receptor, ErbB1. Moreover, the transforming potential and signaling pathways activated by different dimers are distinctive. For example, ErbB1–ErbB2 heterodimers are associated with a more robust signal then ErbB1–ErbB1 homodimers [19]. All four members of the ErbB family have the potential to stimulate the Raf-MEK-ERK protein kinase cascade [17,20,21]. However, phospholipase Cγ binds to specific phosphotyrosines of ErbB1, but not the other ErbB family members. Phospholipase Cγ mediates the formation of diacylglycerol and inositol 1,4,5-trisphosphate second messengers. Furthermore, the regulatory subunit of phosphatidylinositol 3-kinase binds to specific phosphotyrosines on ErbB3 and ErbB4, which leads to enzyme activation. The phosphatidylinositol 3-phosphates lead to the activation of the serine/threonine protein kinase B (Akt).

Heterodimerization of two different members of the ErbB family increases the diversity of ligands recognized by individual receptors. Moreover, heterodimerization of the cytoplasmic domains permits the recruitment of different complements of phosphotyrosine-binding signaling molecules thereby increasing the repertoire of signaling pathways that can be activated by a given receptor [19]. SH2 and PTB domain-containing proteins bind to peptidic phosphotyrosines. SH2 domains ($\approx$100 amino acid residues) bind to distinct amino acid sequences defined by 1–6 residues C-terminal to phosphotyrosine; PTB domains ($\approx$200 amino acid residues) bind to amino acid sequences defined by 3–5 residues N-terminal to phosphotyrosine [1].

The first member of the EGF receptor family is ErbB1, corresponding to the original EGF receptor [17]. ErbB1 is overexpressed in bladder, breast, head and neck, kidney, non-small cell lung, and prostate cancers [9,14,15]. Three truncated forms of the EGF receptor have been described. EGFRvIII (variant III) lacks the majority of the ectodomain and does not bind EGF [17]. This variant had been detected in breast cancer and glioblastoma multiforme (which accounts for about 25% of all primary brain neoplasms in adults). ErbB1, or the EGF receptor, is a protein-tyrosine kinase with a large extracellular ligand-binding domain (622 residues), a transmembrane segment (23 residues), and a large intracellular domain (522 residues) with protein-tyrosine kinase activity (Fig. 1). ErbB1 was the first receptor protein-tyrosine kinase to be sequenced. Ullrich et al. [22] predicted the extracellular, transmembrane, and intracellular topology of the receptor based upon cDNA analysis, and this prediction has stood the test of time.

The second member of the family is ErbB2. No known growth factor binds to ErbB2 homodimers. Rather, ErbB2 forms heterodimers with each of the other family members, and such heterodimers can bind growth factors [19,20]. ErbB2 is overexpressed in breast, cervix, colon, endometrial, esophageal, lung, and pancreatic cancers [9,14,15].

The third member of the family is ErbB3. Although ErbB3 has a tyrosine kinase domain that is highly homologous to those of the other family members, the kinase activity of ErbB3 is impaired [20,23]. ErbB3 can form heterodimers with the other three family members. Owing to the lack of protein kinase activity of ErbB3, *trans* phosphorylation by other members of the EGF receptor family is required for cell signaling. ErbB3 is overexpressed in breast, colon, prostate, and stomach malignancies [9,14,15].

The last member of the family is ErbB4 [21]. The identity of ErbB4 residues with the corresponding residues of the other family members is $\approx$75% in the transmembrane, $\approx$70% in the intracellular catalytic domain (276 residues), and $\approx$20% in the carboxyterminus. The ErbB proteins are homologous to the avian viral oncogene v-ErbB. ErbB4 is overexpressed in breast cancer and granulosa cell tumors of the ovary [9].

### Ligand binding to the ErbB ectodomains and receptor activation

The stoichiometry for epidermal growth factor binding to activated receptor is 2 mol EGF to 2 mol EGFR [24]. This finding is consistent with a mechanism involving each of two EGF molecules spanning the EGF receptor dimer interface (a divalent EGF binding simultaneously to two EGF receptors) or with EGF binding to a single receptor and promoting receptor dimerization (a monovalent EGF binding to a single EGF receptor). The extracellular component of the four family members consists of domains I–IV (Fig. 1) where domains II and IV are cysteine-rich and each contains about 10 disulfide linkages.

Contrary to expectation [24], the X-ray crystallographic structures show that EGF and TGF-α bind to a single receptor and do not span between two receptors [3,25,26]. The chief contacts between receptors involve domain II (Fig. 2). A dimerization arm extends from domain II of a receptor to domain II of an adjacent receptor; there is a reciprocal dimerization arm extending from the adjacent to the first receptor. The dimerization arm consists of residues 242–259 of the EGF receptor. Deletion or mutations of the dimerization arm prevent ligand-induced EGF receptor activation [25,26].

EGF and TGF-α bind to domains I and III [3,25,26]. The bound ligand fixes the relative positions of the two domains. The ligand interacts with the backbone residues of domain I and the R-groups of the amino acids of domain III. The highly conserved arginine (Arg41 in EGF, Arg42 in TGF-α) interacts with a conserved aspartate (Asp355) in the EGF receptor, and a critical leucine (Leu47 in EGF, Leu48 in TGF-α) projects into a well-defined hydrophobic pocket on the domain III surface.

The structure of domains I–IV was established for non-activated receptors corresponding to EGF/EGFR at pH 5 and unliganded ErbB3 [3,27,28]. Ligand binding alters the disposition of domains II and III but not the relative orientation of domains I and II. In the non-activated receptor, domain II binds to domain IV, and the dimerization arm is buried and unable to interact with an adjacent receptor (Fig. 2). The inactive receptor is autoinhibited by this interaction. Moreover, the two ligand-binding sites on domains I and III are too far apart for a single ligand to bind to both simultaneously.

A substantial domain rearrangement is required to switch between the inactive and active conformations of the EGF receptor. Starting from the buried conformation, simultaneous binding of ligand to both domains I and III requires a 130° rotation of the fixed domain I/domain II pair (Fig. 2). This rearrangement gives rise to the extended conformation and breaks the intramolecular domain II/IV linkage, or tether, so that the dimerization arm is free to participate in intermolecular interactions. The investigators responsible for the X-ray structural work on the extracellular domains of the ErbB family suggest that ligand binding traps the extended, or active mode, from the dynamic ensemble of conformations that the receptor can assume [3]. They estimate that 95% of the receptors exist in the inactive and 5% exist in the active conformation [3]. The percentage of molecules in the active conformation is increased by the addition of ligand that binds preferentially to the active form. The EGF receptor consists of low and high affinity forms in a ratio of 95/5 [12]. Perhaps the low affinity form reflects EGF binding to domain I or domain III of the inactive state, and the high affinity form reflects EGF binding to both domains I and III of the active state.
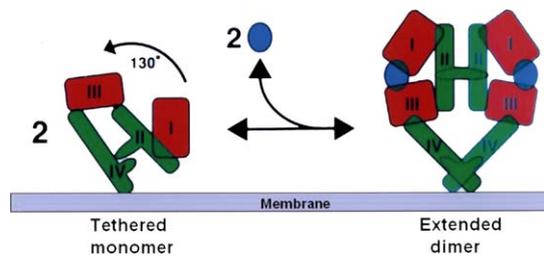


Fig. 2. Ligand induced conformational changes in the EGF receptor ectodomain. The inactive (tethered) EGF receptor ectodomain is shown on the left, and the active form is shown on the right. EGF binding requires a 130° rotation of domains I and II about the axis of the domain II/III junction. This figure is reproduced from [3], with permission of Elsevier.
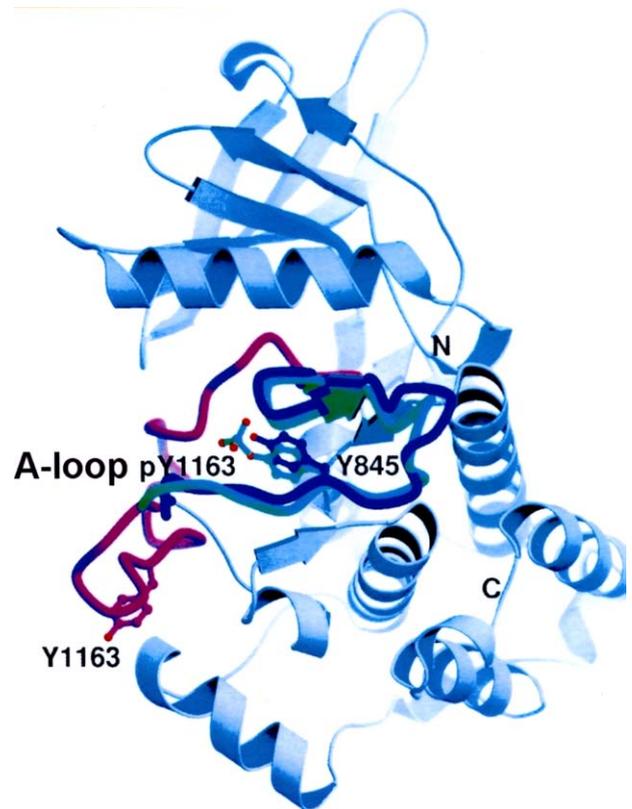


Fig. 3. The bilobed structure of the EGF receptor protein kinase domain. The upper N-terminal lobe contains a major α-helix and a glycine rich ATP-phosphate binding loop, and the lower C-terminal lobe contains the catalytic and activation loops. The activation loop of non-phosphorylated EGF receptor kinase is dark blue, that of the superimposed phosphorylated insulin receptor kinase is green, and that of the non-phosphorylated insulin receptor kinase is red. Y845 is the unphosphorylated tyrosine from the EGF receptor kinase, and this is superimposed in the phosphorylated tyrosine (pY1163) of the insulin receptor kinase in its active conformation. The activation loop of the EGF receptor kinase is fixed in an active conformation despite the lack of phosphorylation. This figure is reproduced from [3], with permission of Elsevier.

## Structure of the ErbB2 ectodomain

The second member of the family, ErbB2/HER2, has several unique properties [3]. First, ErbB2 lacks a

known direct ligand; in order to function, it must work as a co-receptor, or heterodimerization partner, for other ErbB receptors that possess stimulatory ligands [15,19,20]. Second, unlike other ErbB receptors, ErbB2 overexpression can cause malignant transformation without the expression of a growth factor. This observation suggests that ErbB2 has a high level of constitutive (ligand-independent) activity, and ErbB2 expression above a specific threshold can drive tumor growth [15].

X-ray crystallographic structures of the ectodomain of ErbB2 have shed light on its unusual properties [3,29,30]. The ectodomain of ErbB2, in the absence of ligand, resembles the extended conformation of ErbB1 suggesting that ErbB2 is autoactivated. ErbB2 lacks the domain II/domain IV tether. Moreover, three of the seven residues important for stabilizing the tether in ErbB1 and ErbB3 are different in ErbB2. The conformation of ErbB2 reveals an interface between domains I and III that mimics the bridging of these two domains by bound ligand in the active form of ErbB1. The interaction of domains I and III seems to preclude the binding of a small ($\approx$50 residue) peptide ligand. Furthermore, several residues that play important ligand binding roles in ErbB1 are replaced in ErbB2 by residues expected to impair ligand binding (Met10, Arg13, and Pro15 in ErbB2). These features indicate that there is no ErbB2 ligand; the portion of ErbB2 that corresponds to the ligand-binding site of ErbB1 and ErbB3 is mutated and obstructed [3].

The finding that ErbB2 adopts an extended structure with its dimerization arm exposed indicates that ErbB2 is always poised to homodimerize or to form heterodimers with ligand-activated forms of the other ErbB receptors. Biophysical studies fail to detect significant homodimerization of the ErbB2 ectodomain. However, some constitutive homodimerization may occur with the entire ErbB2 molecule involving transmembrane and intracellular components. The ErbB2 ectodomain may be suited to its role as the preferred heterodimerization partner, or co-receptor, for other ErbB receptors [19,20]. ErbB2/ErbB3 heterodimers are the most prevalent and mitogenically potent of the ErbB receptor/ligand complexes [19]. This is paradoxical because one partner (ErbB2) lacks a stimulatory ligand, and the other partner (ErbB3) is kinase dead. Thus far, the structural basis for the prevalent formation of ErbB2/ErbB3 complexes is unknown. In contrast to ErbB1 and ErbB4, ErbB2 and ErbB3 (the impaired receptors) do not form homodimers efficiently.

## Therapeutic antibodies directed to ErbB ectodomains

A search for genetic alterations in breast cancers showed that ErbB2, or HER2 (human EGF receptor 2),

is amplified up to 100-fold in tumor cells from about 25% to 30% of people with invasive breast cancer. A significant clinical correlation exists between HER2 overexpression and the severity of the malignancy [14].

Mouse chimeric humanized monoclonal antibodies targeted against the extracellular component of ErbB2 are clinically useful [31]. Trastuzumab (Herceptin from Genentech) is a humanized mouse monoclonal antibody [32] that was approved for the treatment of ErbB2, or HER2, overexpressing breast cancers in 1998. It is used alone and in combination with other drugs in the treatment of breast cancers that have spread or have the potential to spread to other body sites. This was the first genomic-research based product approved for cancer therapy. HER2 overexpressing breast cancer cells are identified by immunohistochemistry or by fluorescence in situ hybridization (FISH) allowing the selection of people that are likely to respond to trastuzumab therapy. The efficacy of trastuzumab requires the entire antibody molecule with its Fc domain intact, suggesting that antibody-dependent cellular toxicity plays a therapeutic role.

Trastuzumab, a protein, is given during weekly intravenous infusions. About 5% of people receiving this antibody develop congestive heart failure. Heart contains HER2 and HER4 receptors, but the precise mechanism of cardiotoxicity is unknown.

The structure of the trastuzumab Fab fragment bound to the extracellular portion of ErbB2 indicates that its epitope is toward the carboxyterminus of domain IV [30]. The binding site includes the analogous residues of domain IV that participate in the intramolecular tether observed in ErbB1 and ErbB3. Domain IV does not participate in receptor dimerization, and blockade of dimerization does not explain the mechanism of action of the antibody. However, domain IV contains a cleavage site for metalloproteases. Once the extracellular domain is cleaved, the remnant ErbB2 transmembrane and intracellular components dimerize and lead to protein-tyrosine kinase activation [33]. The reduction of ErbB2 signaling and the antibody-dependent cellular cytotoxicity explain, at least in part, the therapeutic action of trastuzumab.

IMC-C225 (cetuximab, or Erbitux, from ImClone) is a humanized mouse monoclonal antibody targeted against the ectodomain of the EGF receptor (ErbB1). This antibody binds to the receptor with a high affinity comparable to that of EGF and TGF-$\alpha$ ($K_D = 0.1$ nM). It prevents ligand binding and receptor activation. IMC-C225 is in clinical trials in patients with head and neck squamous cell cancers and non-small cell lung cancer [34]. IMC-C225 in combination with irinotecan (a DNA topoisomerase I inhibitor) has been approved by the United States Food and Drug Administration for the treatment of colorectal cancer that has spread to other parts of the body. For people with neoplasms that

express the EGF receptor and who no longer responded to treatment with irinotecan alone, the combination treatment of IMC-C225 and irinotecan shrank tumors in 23% of patients and delayed tumor growth by approximately 4 months. For people who received IMC-C225 alone, the tumor response rate was 11% and tumor growth was delayed by 6 weeks.

## Structure of the epidermal growth factor receptor protein-tyrosine kinase domain

The EGF receptor protein-tyrosine kinase domain has the characteristic bilobed architecture observed in all protein kinases (Fig. 3) as predicted by Taylor and co-workers [35]. Residues 685–769 make up the amino-terminal lobe of the kinase, and residues 773–953 make up the carboxyterminal lobe. The smaller lobe has a predominantly antiparallel β-sheet structure. It contains the glycine-rich (GSGAFG) ATP-phosphate binding loop composed of residues 695–700. The large lobe is predominantly α-helical in nature (Fig. 3). The carboxyterminal lobe is responsible for binding the peptide or protein substrate. As described for other protein kinases [35], the catalytic site of the EGF receptor kinase lies in a cleft between the two lobes. Distal to the carboxyterminal lobe is a Leu–Val–Ile (955–957) sequence that plays a role in ligand-independent receptor dimerization. This sequence interacts with the large kinase lobe [4].

Hunter and co-workers [36] identified 12 subdomains with conserved amino acid residue signatures that constitute the catalytic core of protein kinases. Of these, the following three amino acids, which define a K/D/D motif, illustrate the inferred catalytic properties of the EGF receptor kinase. Lys721 of the EGF receptor kinase represents an invariant residue of protein kinases that forms ion pairs with the β- and γ-phosphates of ATP. Asp813, the catalytic base, orients the tyrosyl group of the substrate protein in a catalytically competent state and may abstract a proton from tyrosine thereby facilitating its nucleophilic attack of the γ-phosphorus atom of MgATP. Asp831 is the first residue of the activation loop found in the large lobe. Asp831 binds $Mg^{2+}$, which in turn coordinates the β- and γ-phosphate groups of ATP.

Divalent cations play differing roles in protein kinase activity. Protein kinase A binds ATP with one or two $Mg^{2+}$ ions. The first ion binds with high affinity to the β- and γ-phosphate groups of ATP; the second ion binds with lower affinity and coordinates the α- and γ-phosphate groups of ATP [37]. All protein kinases require binding by the first metal ion for activity. The consequences of the second ion vary. Binding the second ion reduces the $k_{cat}$ of protein kinase A, but the $K_m$ is decreased [38]. ErbB1, ErbB2, and ErbB4 have optimal $Mg^{2+}$ concentrations far above the concentration bound to ATP; this suggests that $Mg^{2+}$ occupancy of the second site is required for optimal activity [39].

The catalytic loops surrounding the actual site of phosphotransfer are different between the protein-serine/threonine and protein-tyrosine kinases [35]. This loop is made up of RYDLKPEN in protein serine/threonine kinases and HRDLAARN in protein-tyrosine kinases including the EGF receptor (His811–Asn 818), ErbB2 (822–829), and ErbB4 (816–823). In contrast, ErbB3 contains the sequence HRNLAARN (813–820). Thus, the catalytic base (aspartate) is converted into an asparagine (N815) yielding a catalytically dead kinase [23]. The AAR sequence in the catalytic loop represents a receptor protein-tyrosine kinase signature, and RAA represents a non-receptor protein-tyrosine kinase signature.

## The activation loop

The conformation of the activation loop differs between activate and dormant kinases [40,41]. The activation loop of nearly all protein kinases begins with DFG and ends with APE. That of the EGF receptor kinase begins with DFG (831–833), but ends in ALE (858–860). In protein kinases that are dormant, the activation loop has a compact conformation that inhibits the binding of protein substrates (and ATP in some kinases). In most protein-tyrosine kinases, a tyrosine residue in the activation loop plays a regulatory role. When the activation loop is in a compact conformation, the hydroxyl group of the non-phosphorylated tyrosine forms a hydrogen bond with the catalytic aspartate thereby preventing the binding of protein substrates to the active site. When the activation loop is in an open conformation, the tyrosine residue is accessible and can be phosphorylated in *trans* by its dimer partner [1]. This phosphorylation stabilizes the open conformation of the activation loop and maintains the enzyme in its active form [40,41].

The EGF receptor protein kinase is an exception that does not follow the general activation-loop phosphorylation mechanism [4]. The EGF receptor kinase contains a tyrosine residue in its activation loop; however, this residue does not become phosphorylated by its dimer partner [12]. Moreover, mutation of this tyrosine to a phenylalanine does not affect ligand-dependent enzyme activation [42].

The unphosphorylated activation loop of the EGF receptor assumes an open and active conformation. In contrast, the unphosphorylated insulin receptor activation loop occurs with a compact conformation, and the phosphorylated insulin receptor activation loop occurs in an open conformation as illustrated in Fig. 3 [40]. The activation loop of the EGF receptor contains four glutamate residues (EEKEYHAE, 841–848), and these negatively charged groups might obviate the need for a negatively charged phosphate in the activation loop [4].

## The protein kinase inhibitory ectodomain of ErbB proteins

Two lines of evidence indicate that the ectodomain constrains EGF receptor kinase activity. First, the v-erb murine oncoprotein, homologous to the EGF receptor, is a constitutively active protein kinase that consists of a short ectodomain, a transmembrane segment, and an intracellular catalytic domain [13]. The ectodomain does not bind ligand. Furthermore, the human oncoprotein (EGFRvIII) that lacks residues 6–273 is unable to bind EGF and is constitutively active [17]. These findings indicate that the ectodomain maintains the enzyme in a catalytically constrained conformation.

When the ectodomain is truncated, absent, or binds to ligand, it no longer inhibits kinase activity. This may explain in part why the EGF receptor kinase domain, in the absence of the inhibitory ectodomain, is found in the active state. Whether the dormant state of the entire protein (not the kinase domain alone) in the absence of EGF has the open or closed activation loop conformation remains to be established. It is clear that the unphosphorylated form of the kinase domains of the insulin and insulin-like growth factor receptors assume an inactive conformation while that of the EGF receptor assumes an active conformation, but the reason for this difference is uncertain.

## Inhibition of the EGF receptor protein-tyrosine kinase by anilinoquinazolines

Inhibition of the EGF receptor kinase has been proposed as a rational approach to cancer therapy for about 15 years. The identification of 4-anilinoquinazolines ushered in a new era owing to the potency for this enzyme [43]. These compounds are reversible competitive inhibitors with respect to ATP.

Two drugs that have emerged as therapeutic EGF receptor kinase inhibitors include ZD1839 and OSI-774 (Fig. 4), both of which are active in humans when given orally. The antineoplastic effects are mediated by several mechanisms including induction of cell-cycle arrest and apoptosis. A common side effect for both drugs is the occurrence of acneiform skin lesions [43]. The epidermal skin lesions suggest that the drugs are effective in blocking the EGF receptor, but they do not indicate directly whether the drugs are producing the desired effects in tumors.

ZD1839 (gefitinib, or Iressa, from AstraZeneca) has been approved for the treatment of non-small cell lung cancer in the US and in Japan. It is used as monotherapy for the treatment of patients with locally advanced or widely spread non-small cell lung cancer after failure of standard chemotherapies. ZD1839 has an $IC_{50}$ value for the EGF receptor kinase of 33 nM in vitro and 80 nM in a cell-based assay [44]. Besides non-small cell lung ca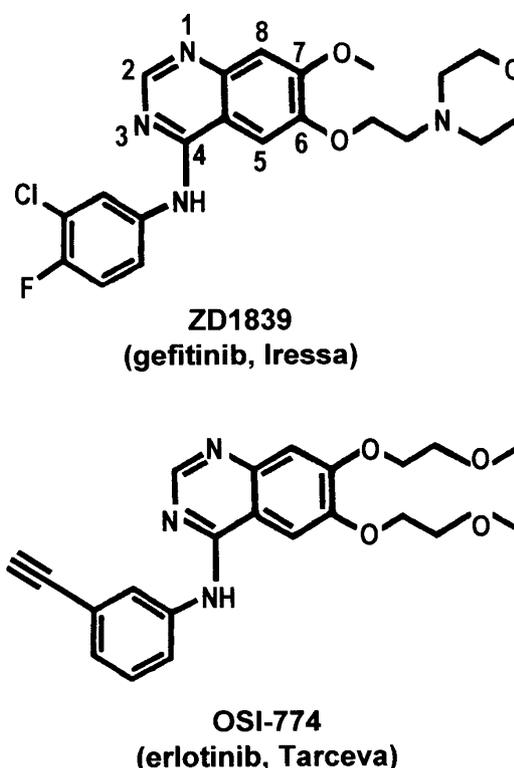ncer, ZD1839 is being tested in combination with other drugs in people with other solid tumors including colorectal cancer. Although this drug is said to be a specific inhibitor of the EGF receptor kinase activity and not other protein kinase activities, there are little data in the literature that documents this selectivity [45].

OSI-774 (erlotinib, or Tarceva, from OSI Pharmaceuticals, Roche, and Genentech) is another 4-anilinoquinazoline that inhibits the EGF receptor protein-tyrosine kinase. It has not yet received Federal Drug Administration approval, but it is in phase III trials for non-small cell lung cancer. OSI-774 has an $IC_{50}$ value for the purified EGF receptor of 2 nM and 20 nM for this enzyme in intact cells [46]. The $IC_{50}$ values of both ZD1839 and OSI-774 for ErbB2 are more than 100-fold higher than the corresponding value for ErbB1.

## Binding of OSI-774 to the EGF receptor protein kinase domain

The X-ray crystallographic structure of the catalytic domain of the human EGF receptor protein-tyrosine kinase domain has been solved in the presence and absence of OSI-774 [4]. The drug lies with the N1 and C8-containing edge of the quinazoline directed toward the peptide segment connecting the N-terminal and C-terminal lobes of the kinase. The N1 of the quinazoline



Fig. 4. Structures of ZD1839 and OSI-774. The quinazoline numbering convention is indicated.

accepts a hydrogen bond from the Met769 amide nitrogen (Fig. 5); this is how the N1 of ATP is inferred to bind to the enzyme. Replacement of N1 with carbon decreases the inhibitor potency for ErbB1 by 3700-fold indicating the importance of this hydrogen bond. The other quinazoline nitrogen atom, or N3, is not within hydrogen bonding distance of the Thr766 side chain, and a water molecule bridges the gap. Replacement of N3 with carbon decreases inhibitor potency by 200-fold. The C2 of the quinazoline ring is 3.1 Å from the carbonyl oxygen of Gln767 (not shown), and the C8 is 3.2 Å from that of Met 769. Substitution of the hydrogens at C2 or C8 with methyl or methoxy groups abolishes enzyme inhibition [43]. The effect of these unfavorable substitutions may result from steric hindrance.

The 4-aniline group plays an important role in the affinity of the kinase for these inhibitors. The interplaner angle of the OSI-774 aromatic ring systems is 42° when bound to the enzyme (Fig. 5), and this directs the acetylene moiety into a pocket that many kinase domains share when the amino acid at position 766 is small (Thr766 in EGFR). This region, called the hydrophobic pocket, is formed by side chains of several residues (Thr766, Met742, and part of Lys721). OSI-774, but not ATP, binds to this pocket.
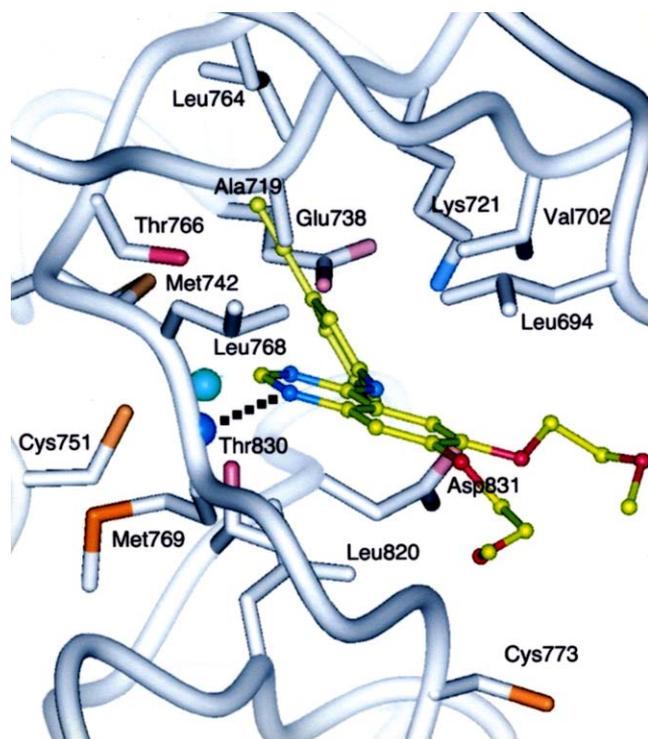


Fig. 5. Binding of OSI-774 to the EGF receptor protein-tyrosine kinase domain. The dashed line indicates a hydrogen bond from the blue Met769 amide nitrogen to N1 of OSI-774. The light blue circle abutting the backbone is a water molecule. The figure is reproduced from [4], with permission of the Journal of Biological Chemistry.

## ErbB glycoprotein synthesis and degradation

Each of the ErbB proteins is an N-linked glycoprotein; about 20% of the mass of these proteins is carbohydrate. When glycosylation of the EGF receptor is blocked by tunicamycin during synthesis in cell culture, the EGF receptor protein kinase activity is only 30% of that of the control receptor [47]. This treatment inhibits the attachment of carbohydrate, and the molecular weight is decreased from 170,000 to 140,000. When carbohydrate is cleaved enzymatically from the mature receptor, protein kinase activity is not diminished. These studies indicate that glycosylation facilitates proper folding of the full-length receptor to generate an active EGF-binding conformation, and once such a conformation is attained, the carbohydrate chains are dispensable.

When EGF binds to its receptor, Stanley Cohen showed that the ligand–receptor complex undergoes endocytosis [2]. This is associated with lysosomal degradation of both EGF and its receptor. The half-life of the EGF receptor in the absence of EGF in cell culture is 6.5 h, and it is 1.5 h in the presence of EGF [48]. These findings led to the study of the downregulation of a variety of receptors. Cells expressing ErbB2/ErbB3 or ErbB4, in contrast to EGFR, are not downregulated by stimulatory ligands [48]. Although members of the same family, each receptor and each kinase has its own distinguishing features [39].

## Epilogue

The EGF receptor is one of the first proteins, if not the first, that was implicated in the production of human malignancies. Neoplasms that are often associated with dysregulation of this receptor include cancers of the lung, colon, and breast. These neoplasms are among the most prevalent malignancies in the US. If inhibitors of the EGF receptor were efficacious in only 10% of these disorders, the number of new treatable cases would be more than 50,000 annually in the US. A major difficulty that has not been overcome, however, is the identification of biomarkers that indicate tumor sensitivity to EGF receptor antagonists. The identification of such an indicator or indicators would be invaluable.

The pathogenesis of nearly all malignancies is the result of multiple somatic cell mutations [49]. In the case of lung cancer, proteins other than the EGF receptor that have been reported to participate in the oncogenic process include myc, K-ras, cyclin D1, Bcl2, p53, and p16 (a cyclin-dependent kinase inhibitor) [50]. As a consequence, it is less likely that these disorders will respond dramatically to a drug that supposedly targets a single oncoprotein such as ErbB1. Nevertheless, a large number of laboratories are developing inhibitors of

protein kinase drug targets including the ErbB family. The use of a combination of cytotoxic drugs to treat malignancies is commonplace, and it will be a challenge to develop combinations of drugs that are targeted toward multiple oncoproteins. The therapeutic goal with targeted drugs is to convert a potentially lethal malignancy into an indolent or chronic disease using protein kinase inhibitors.

A potential advantage of targeted kinase inhibitors is that the side effects are generally less severe when compared with traditional cytotoxic drugs or radiation. If there is a subset of malignancies that are due to augmented EGF receptor action, these may respond favorably. Many neoplasms express both ErbB1 and ErbB2 [14]. The development of a single agent that targets both of these activities or using a combination of ErbB1 and ErbB2 inhibitors has therapeutic potential.

The initiation of chronic myelogenous leukemia differs from that of lung, colon, breast, and most other malignancies. Abl kinase activity is elevated in chronic myelogenous leukemia as a result of a reciprocal chromosomal translocation that leads to the formation of the Philadelphia chromosome and a constitutively active chimeric Bcr-Abl non-receptor protein-tyrosine kinase [7]. Chronic myelogenous leukemia is unusual in that the leukemic state is, at least initially, the result of a single change that produces the Bcr-Abl oncoprotein and is not the result of several somatic cell mutations. STI-571 (imatinib or Gleevec) is an Abl protein-tyrosine kinase ATP competitive inhibitor, which is effective in the treatment of the chronic phase of this disorder [7].

The ATP-binding site of protein kinases was initially thought to be a poor drug target because of the large number of protein kinases and other enzymes that use ATP as substrate and the perceived difficulty in designing specific inhibitors. Moreover, the concentration of cellular ATP is 1–5 mM, and the $K_m$ of many protein kinases for ATP is 0.2–20 µM. The high ratio of the concentration of ATP to its $K_m$ value raised the possibility that inhibitors binding to the ATP site of protein kinases would be ineffective in vivo. However, the effectiveness of STI-571 in the treatment of chronic myelogenous leukemia has dispelled this notion. The greater $IC_{50}$ concentration required to inhibit protein kinase activity in cells compared with that in vitro is due in part to the higher ATP concentration in cells than is traditionally used in enzyme assays. However, the ability of ATP to bind to protein kinases in their inactive conformation is less than that required for enzymes in their active conformation. This difference decreases the competition between ATP and STI-571 (which binds preferentially to the inactive enzyme conformation) and increases the efficacy of STI-571 under physiological conditions. Thus, it may be advantageous to design ATP binding site inhibitors that combine preferentially with the inactive protein kinase conformation.

Besides v-Abl and the platelet-derived growth factor receptor, STI-571 is an inhibitor of c-Abl, Bcr-Abl, and c-Kit (stem-cell factor/mast-cell factor receptor) protein-tyrosine kinases. It is not a potent inhibitor of about 30 other protein kinases that were tested [7]. Moreover, only about 30 of the more than 500 protein kinases have been tested for STI-571 inhibition and reported in the open literature. Even less has been reported on the protein kinase inhibitory profiles of ZD1839 [45] and OSI-774, and such data would provide information on the efficacy, toxicity, and mechanism of action of these compounds. The use of drugs such as ZD1839, OSI-774, STI-571, and other kinase inhibitors as affinity-chromatographic ligands for proteins found in cell extracts may show that these agents bind to more protein kinases than is currently appreciated. The knowledge of the actual kinase profile may help in understanding the pharmacology of these new drugs.

The determination of the tertiary structures of the inactive and activate ectodomains of ErbB family members has provided new insight into the mechanism of receptor dimerization. The intramolecular interactions between domains II and IV constrain dimerization. Growth factor binding to domains I and III releases the inhibition and allows the formation of a receptor dimer. Limiting the location of receptors to cell membranes increases the effective receptor concentration and increases the probability of two receptors combining to form a dimer. The EGF receptor has a tyrosine in the activation loop, but this enzyme does not follow the activation-loop phosphorylation mechanism that is common in other protein-tyrosine kinases. It is unclear how EGF receptor dimerization leads to kinase activation. A similar activation-loop phosphorylation mechanism involving serine or threonine occurs in protein-serine/threonine kinases.

Kennedy and Burnett [51] first demonstrated protein-serine/threonine kinase activity in rat hepatoma extracts in 1954, and Hunter and Sefton [8] first demonstrated (non-receptor) protein-tyrosine kinase activity in v-Src in 1980. Subsequently, Cohen and Chinkers [10] found protein-tyrosine kinase activity in the EGF receptor in 1981, the first demonstration of a receptor protein-tyrosine kinase. Cohen's work began with the discovery of a factor present in submaxillary salivary gland extracts that induced precocious eyelid opening and tooth development in newborn mice [2]. He isolated EGF, determined its sequence, studied its binding to the EGF receptor, and showed that a single protein molecule contained EGF binding and protein kinase activity. He also showed that EGF and its receptor are taken up by cells and are degraded in lysosomes [2]. It is inconceivable that anyone could have imagined that the discovery of a "tooth-lid" factor would lead eventually to a more complete understanding of the growth behavior of malignant cells.

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