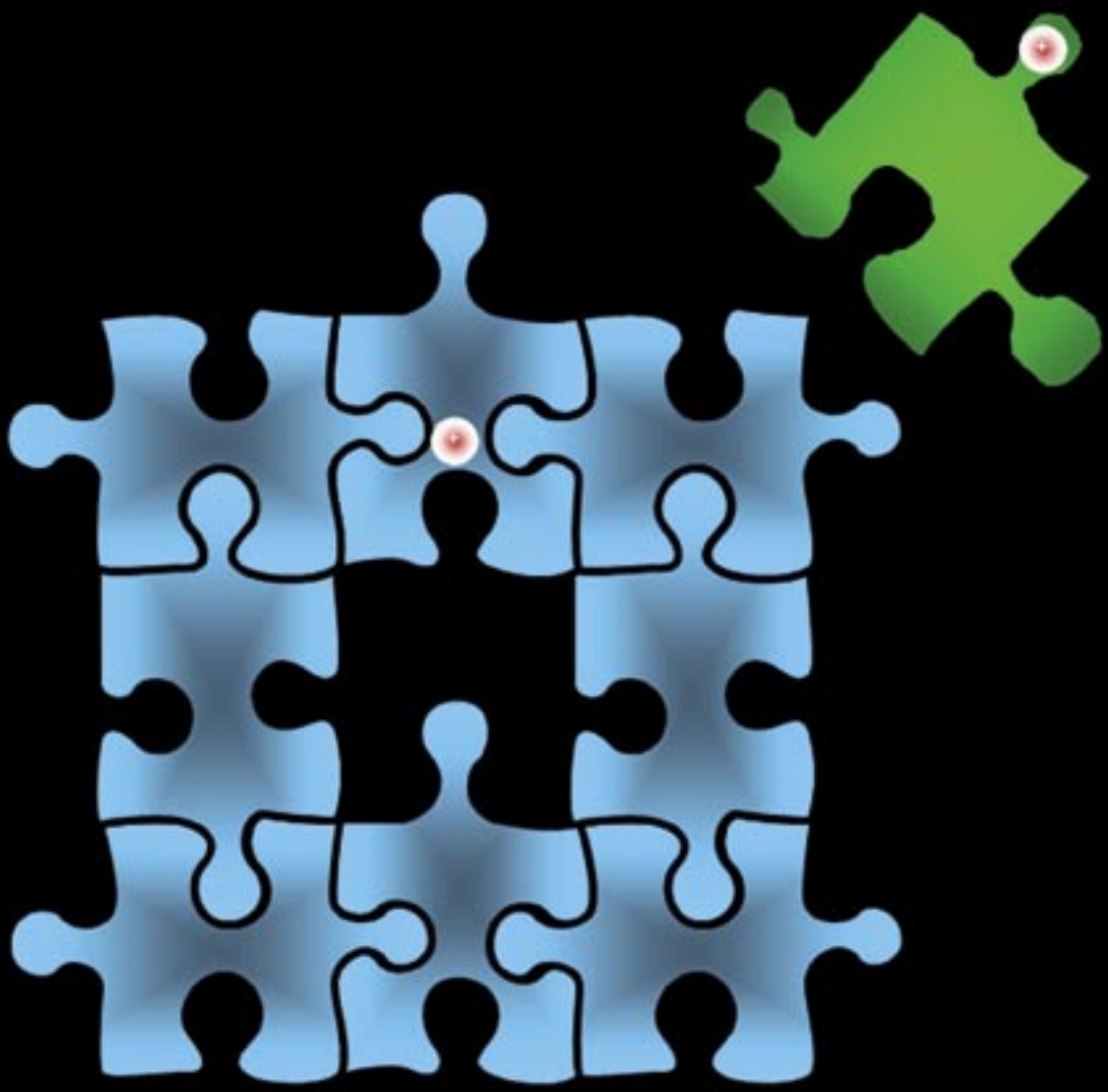
Molecular mechanisms that underlie the receptor specificity of ErbB ligands



Sebastian van der Woning

Molecular mechanisms that underlie the receptor specificity of ErbB ligands

Printed by
PrintPartners Ipskamp, Enschede

ISBN: 978-90-9024963-6

The printing of this thesis was financially supported by the Radboud University Nijmegen

Cover: ‘What seems to be a perfect fit is prevented by subtile obstacles’

Molecular mechanisms that underlie the receptor specificity of ErbB ligands

een wetenschappelijke proeve op het gebied van de
Natuurwetenschappen, Wiskunde en Informatica

Proefschrift

ter verkrijging van de graad doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op dinsdag 12 januari 2010
om 13.30 uur precies

door

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geboren op 6 maart 1974
te Borne

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General Introduction

1 Introduction

The development of a fertilized egg to an adult organism involves a complexity of growth and differentiation processes which are controlled by a.o. polypeptide growth factors and their cellular receptors. One of the best studied growth factor signaling system is the ErbB network, which is involved in the development of most organs in the multicellular organism. The family consists of four ErbB receptors and eleven epidermal growth factor (EGF)-like ligands, which are differentially expressed throughout the body with expression levels that strongly depend on the stage of development (1). Aberrant expression of ErbB receptors and their ligands can cause cancer. Gene amplification of the ErbB1 and ErbB2 genes is frequently observed in breast cancers and correlates with chemotherapy resistance and poor patient prognosis (2-4). The ErbB receptors are therefore main targets for the development of anti-cancer drugs and currently several tyrosine kinase inhibitors and anti-ErbB antibodies have received FDA approval for therapeutic application.

Crystal structures of both ligand-bound and unoccupied ErbB receptors have greatly increased our knowledge about the process whereby ligand binding induces receptor dimerization (5-9). In combination with mutation and domain-exchange studies, such crystal structure analyses have identified the amino acids which are directly involved in high affinity ErbB-ligand interaction. However, the molecular basis for receptor selectivity and mitogenic activity of the ErbB ligands is less well understood. This thesis focusses mainly on amino acids in ligand molecules that prevent unintended ErbB binding, and thereby determine the receptor specificity of EGF-like growth factors. Knowledge of the basis of high affinity binding, specificity and mitogenic activity are very valuable in the design of therapeutic ErbB ligands with designed properties, both agonistic and antagonistic.

2 ErbB receptors and their ligands

The family of ErbB ligands consists of polypeptide growth factors that all share an EGF-like domain, which is essential and sufficient for binding to and activation of ErbB receptors. This domain is characterized by a conserved spacing of six cysteines that make up three disulphide bridges. As a result EGF-like growth factors are composed of an N-terminal linear region, an A-loop (1^{st}–3^{rd} cysteine), B-loop (2^{nd}- 4^{th} cysteine), a hinge region (4^{th}–5^{th} cysteine), a C-loop (5^{th}–6^{th} cysteine), and a C-terminal linear (Figure 1). Mammals contain 11 genes for EGF-like growth factors, some of which give rise to multiple isoforms. The average length of the EGF-like domain is about 50 amino acids. Figure 2 shows the amino acid sequence of the EGF-like domain of human ErbB ligands, as well as of related viral and *Drosophila* proteins. The ligands bind the four ErbB receptors in a highly selective manner, such that EGF,

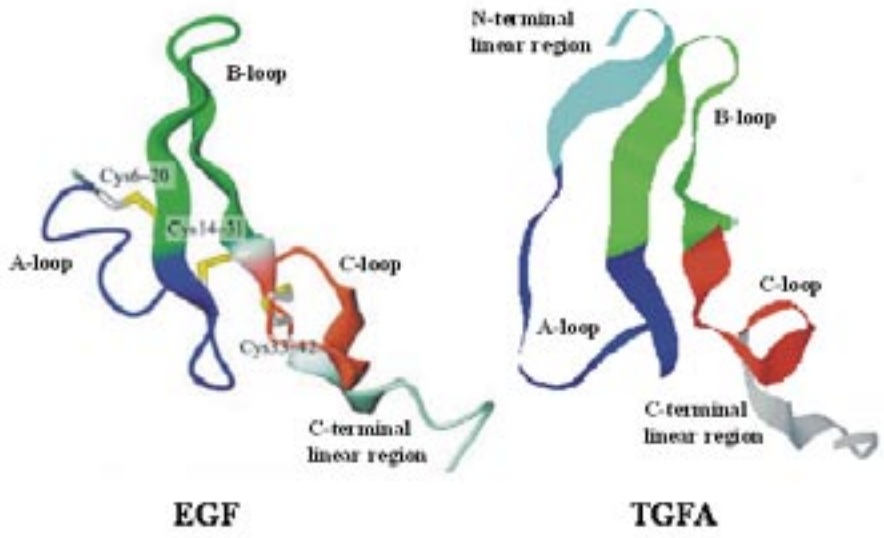


Figure 1. The EGF domain. These ribbon structures of EGF and TGFA represent crystal structures. The unstructured N-terminal linear region of EGF is not depicted. The various domains are indicated. Figure taken from Ogiso et al. (7).

transforming growth factor alpha (TGFA), amphiregulin (AREG) and epigen (EPGN) form a subfamily that binds selectively to ErbB1, while the subfamily consisting of betacellulin (BTC), epiregulin (EREG) and heparin-binding EGF (HBEGF) binds not only ErbB1, but also ErbB4. Finally the neuregulins form a subfamily of which NRG1 and NRG2 bind both ErbB3 and ErbB4, while NRG3 and NRG4 are specific for ErbB4. ErbB2 does not bind ligand by itself, but is the preferred heterodimerization partner for all liganded ErbBs.

In their mature form, the ligands EGF and TGFA consist only of an EGF-like domain, whereas the ligands HB-EGF, AREG and BTC have in addition a hydrophilic Arg-Lys rich region N-terminally of the EGF-like domain, which allows them to bind to heparan-sulphate groups present in the extracellular matrix. As a result of alternative splicing and multiple promoter use, the NRG1 and NRG2 genes give rise to many distinct isoforms. As a consequence, at least 15 different isoforms are transcribed from the NRG1 gene (reviewed in (10)). The α and β isoforms of NRG1 and NRG2 differ in the EGF-domain sequence C-terminally of the fifth cysteine (see Figure 2). The so-called Type I, II and III isoforms differ in their N-terminal sequence, whereby Type II has an enlarged N-terminal region and Type III lacks an Ig-like domain but has an additional transmembrane domain. Some of these isoforms are initially synthesized as a transmembrane protein whereas others are secreted. The Ig-like domains, present in some of the NRG isoforms, interact with heparin-sulphate proteoglycans to maintain high NRG concentrations at sites where

Figure 2. Sequence alignment of EGF-like ligands. All sequences are human except for the Drosophila growth factors Spitz, Keren, Gurken, Vein and Argos, and the viral growth factors vaccinia virus growth factor (VGF), Shope fibroma virus growth factor (SFGF) and myxoma virus growth factor (MGF). Residue numbers are according to EGF, of which the conserved cysteines are indicated.

sustained ErbB receptor activation is required, for example in the neuromuscular junction (11).

The four ErbB receptors consist of an extracellular domain, a single transmembrane domain and an intracellular domain. The extracellular domain of the ErbB receptors can be subdivided in four subdomains; two ligand binding subdomains (L1 and L2) and two cysteine-rich subdomains (S1 and S2) (Figure 3). In some literature these extracellular subdomains are also numbered I to IV. The intracellular domain contains the tyrosine kinase region as well as the docking sites for signaling molecules and adaptor proteins. ErbB2 is an orphan receptor that is unable to bind ligand, while ErbB3 has impaired tyrosine kinase activity.

3 Evolution of ErbB receptors and their ligands

During evolution ErbB receptors have remained conserved among many species and have been evolved from a single progenitor receptor (12). In invertebrates, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, only a single receptor type has been identified. In mammals there are four family members, ErbB1 (EGFR), ErbB2 (neu), ErbB3 and ErbB4. Two receptors, resembling ErbB1 and ErbB4 homologues, have been

identified in the ascidian *Ciona intestinalis*. Parallel to the evolution of the receptors their ligands have evolved: whereas the *C. elegans* ErbB receptor has only one ligand, Lin-3, the *Drosophila* EGF receptor (DER) has four ligands Spitz, Keren, Vein, and Gurken. The mammalian ErbB-ligand system is much more complex: the four receptors are bound by a set of eleven ligands. Comparison of vertebrate ErbB receptors suggests that a gene duplication event generated two ancestral receptors, an ErbB3/ErbB4 precursor and an ErbB1/ErbB2 precursor. Phylogenetic analysis suggests that the mammalian ligands initially segregated into ErbB1-specific and ErbB3/ErbB4-specific ligands (12, 13). Indeed, EGF and TGFA interact with high affinity exclusively to ErbB1, while the neuregulins bind only ErbB3 and ErbB4. However, some of the ErbB ligands show dual specificity, including betacellulin (BTC), heparin binding EGF-like growth factor (HB-EGF) and epiregulin (EREG), which interact with both ErbB1 and ErbB4. Phylogenetic analysis shows that TGFA and BTC, as well as AREG and HB-EGF and also EREG and epigen (EPGN) have shared ancestors. It therefore appears that after the second gene duplication event each of the ligand pairs developed into an ErbB1-specific ligand and a ligand that can interact with both ErbB1 and ErbB4. Apparently, ligands and receptors have coevolved while putting evolutionary pressure on each other. Since no ligands have been identified that directly interact with ErbB2 and because ErbB2 is always in an active conformation, it is reasonable to assume that ErbB2 has evolved as a dimerization partner for other the ErbB receptors. The possibility to form heterodimers with the kinase-inactive ErbB3 receptor could have put evolutionary pressure on ErbB2 for maintenance of kinase activity and allowed ErbB3 to gain multiple PI3-kinase binding sites (12).

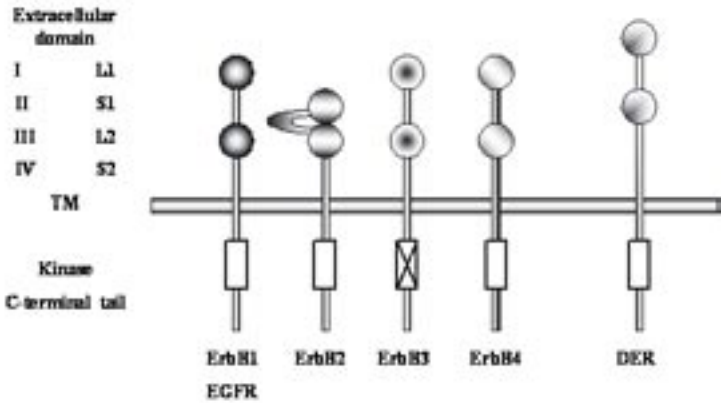
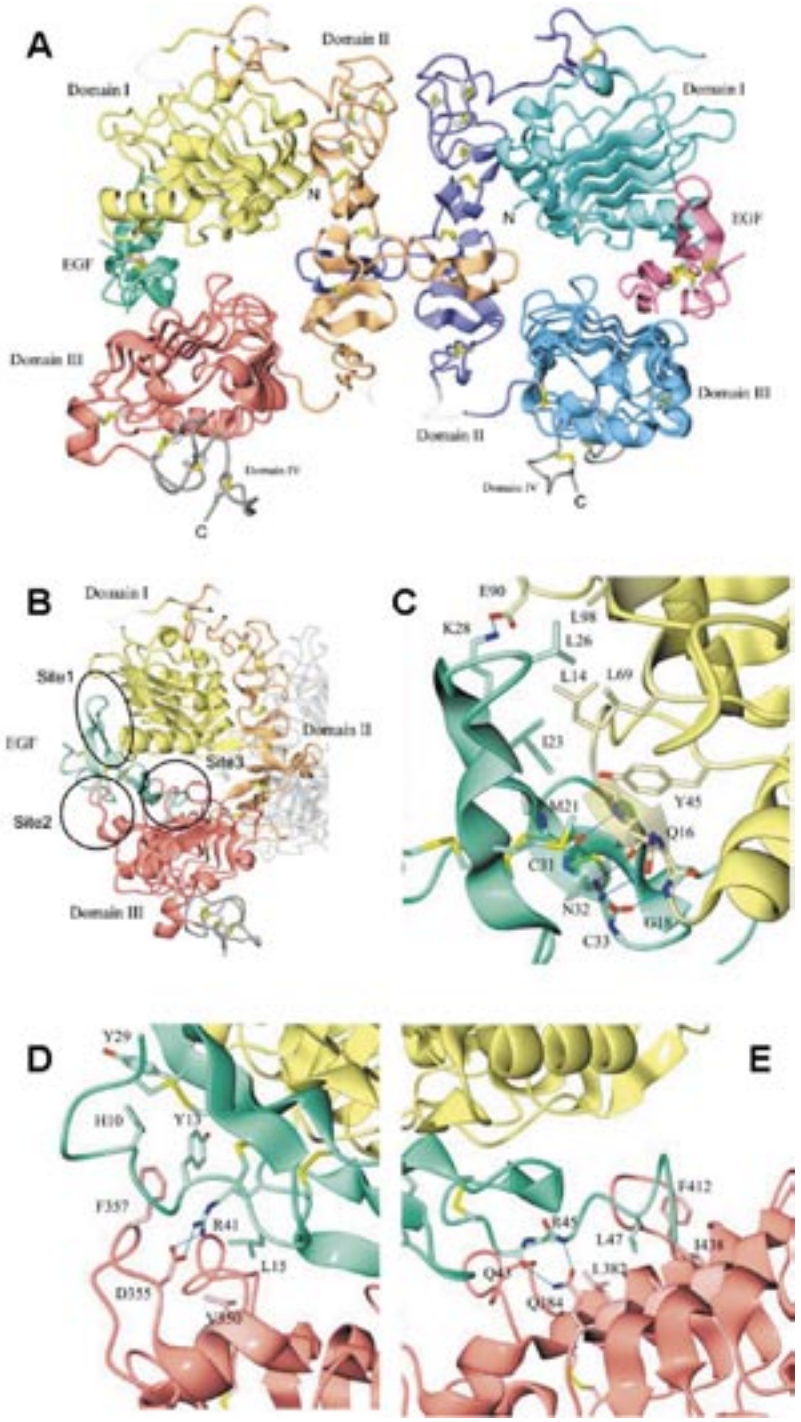


Figure 3. Schematic representation of the mammalian ErbB family of receptor tyrosine kinases and the Drosophila epidermal growth factor receptor. The extracellular domains of the receptors consist of two leucine-rich ligand binding domains (L1 and L2, represented by balls) and two cysteine-rich dimerization domains (S1 and S2). In ErbB2 the major dimerization interface, S1, is continuously exposed for dimerization. The Drosophila epidermal growth factor receptor (DER) has an elongated S2 domain.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Domain II
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Domain I
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C
B
Domain I
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EGF
Domain II
Site3
Site2
Domain III
C
E90
K28
L98
L26
L14
L69
I23
M21
Y45
Q16
C31
N32
G18
C33
D
Y29
H10
Y13
F357
R41
L15
D355
V350
E
F412
R45
L47
I438
Q43
L382
Q384

Figure 4. Crystal structure of ErbB1 in complex with EGF. A. Ribbon representation of the extracellular domain of the EGF-bound ErbB1 dimer. **B.** Three interaction sites by which the ligand binds its receptor. **C.** Close-up of the interaction site 1, consisting of the B-loop, the hinge region and the N-terminal linear region. The main interacting residues are I23 (L23 in TGFA) and L26 of EGF which can bind to the hydrophobic pocket in L1 formed by L14, L69 and L98. TGFA has a Glu (E26) instead of a Leu on position 26, which forms a salt bridge with R124 of ErbB1. K28 forms a salt bridge with E90 in the receptor while C31, N32 and C33 together interact with Q16, L17 and G18 in the receptor by forming a parallel β-sheet. In addition N32 forms a hydrogen bound with Q16 of the receptor. **D.** Close-up of the interaction site 2, in which R41 forms a salt bridge with D355 in the L2 domain of the receptor. This salt bridge is enforced by Y13 (F13 in TGFA) and L15 (F15 in TGFA) of the ligand, which interact with F357 and V350 in ErbB1, respectively. Due to this hydrophobic environment R41 is pointed out into the proper orientation to facilitate salt bridging with D355. **E.** Close-up of the interaction site 3, which includes the C-terminal linear region. The key residue in this interaction is L47, which binds into a hydrophobic pocket formed by L382, A415, V417, F412 and I438 of ErbB1. In addition, Q43 and R45 in EGF form hydrogen bonds with Q384 in the receptor, while E43 and H44 in TGFA form hydrogen bonds with Q384 and Q408 in ErbB1. Figure taken from Ogiso et al. (7).

4 Ligand-mediated receptor dimerization

Recent studies on the crystal structure of ErbB1 in complex with EGF and TGFA, as well as the crystal structures of ErbB2 and unliganded ErbB3 and ErbB4, have provided a breakthrough in our understanding of ligand-mediated receptor activation. The L1 and L2 domain are directly involved in ligand binding (7, 14), while the S1 and S2 domain are involved in receptor dimerization. As shown in Figure 4A, ligand binding results in the formation of a 2:2 ErbB1-ligand complex (7, 14).

The residues in EGF that directly interact with its receptor are located in three sites (Figure 4B). Site 1 contains the B-loop, the hinge region (C31, N32 and C33) and the N-terminal linear region, which all interact with the L1 domain of ErbB1 (Figure 4C). Site 2 contains R41 which forms an essential salt bridge with D355 in the L2 domain of the receptor (Figure 4D), while site 3 contains the C-terminal linear region which interacts with the L2 domain (Figure 4E). More details are provided in the legend of Figure 4.

Receptor dimerization occurs mostly through interactions between the S1 domain (Figure 5A, B and C), which forms the major dimerization interface. A dimerization arm, formed by a 20 amino acid β-hairpin, protrudes from the S1 domain of both receptors. The tip of the arm forms a hand-like structure that interacts with the arm of the other receptor. Mutation studies indicate that the S2 domain may provide a minor dimerization interface, but no crystallographic data are available to confirm this. However, the S2 domain plays a central role to keep the receptor in an auto-inhibited (tethered) conformation in the absence of ligand. Such a conformation has

been visualized in the crystal structure of unliganded ErbB3 (8) and ErbB4 (9), whereas as similar structure was observed for EGF-bound ErbB1 at low pH conditions whereby the receptor cannot dimerize (6). Remarkably, the same β-hairpin loop that is involved in receptor dimerization also forms the tether by folding back to the S1 domain. Key residues in this interaction are described in the legend of Figure 5. Mutation of Y246 in the S1 domain ErbB1, one of the key residues involved in both maintenance of the auto-inhibited conformation and receptor dimerization, had hardly any effect on EGF binding, but abolished EGF-induced receptor dimerization, phosphorylation and MAPK activation. On the other hand, mutations in the S2 domain which also disrupted the intramolecular tether did not affect EGF-induced receptor activation (15).

Binding of activating ligands to the ErbB receptor is accompanied by a dramatic rearrangement of its extracellular domain. Upon ligand binding S1 will dissociate from S2, and the subsequent rotation will bring the ligand binding domains L1 and L2 close together, such that a single ligand molecule can bridge the two domains, while in addition bringing the dimerization interface in the exposed conformation.

Reports about the homodimerization of ErbB2 and ErbB3 are contradictory. Although homodimers of ErbB2 has been described in ErbB2 overexpressing breast cancer cells (16), ErbB2 and ErbB3 do not generally form homodimers. Chimeric ErbB receptors comprising of an extracellular ErbB3 domain and a intracellular ErbB1 (ErbB3/ErbB1) failed to form signaling dimers upon stimulation with NRG, while in presence of ErbB2 signaling ErbB3/ErbB1-ErbB2 heterodimers were formed (17). These findings demonstrate that the ErbB3 extracellular domains are not able to interact with each other. ErbB2 is prevented from homodimerization by charge repulsion since its dimerization interface is highly negatively charged (5). Furthermore, Citri et al. (18) proposed a model in which HSP90 interacts with the ErbB2 kinase domain, thereby keeping putative ErbB2 homodimers in an inactive state, while ligand-induced heterodimerization disrupts the interaction between HSP90 and ErbB2.

Besides S1 and S2, also the transmembrane and juxtamembrane domain appear to be involved in receptor dimerization. The transmembrane domains of the ErbB receptors contain two dimerization motives, a N-terminal and a C-terminal motif, corresponding to an active and an inactive state, respectively. Based on computational analysis of the transmembrane domains, ErbB2 dimerization would be mediated by the C-terminal motif, resulting in an inactive ErbB2 homodimer. From this inactive state the ErbB2 homodimer might rotate into the active state, mediated by the N-terminal motif. Moreover, Moriki et al. (19) analysed receptor dimerization by inserting cysteine residues at strategic positions in the putative α-helix axis of the extracellular juxtamembrane region. Depending on the position of the inserted cysteine the mutant receptors spontaneously formed disulphide bridges. These results suggest that ligand binding to the flexible extracellular domains

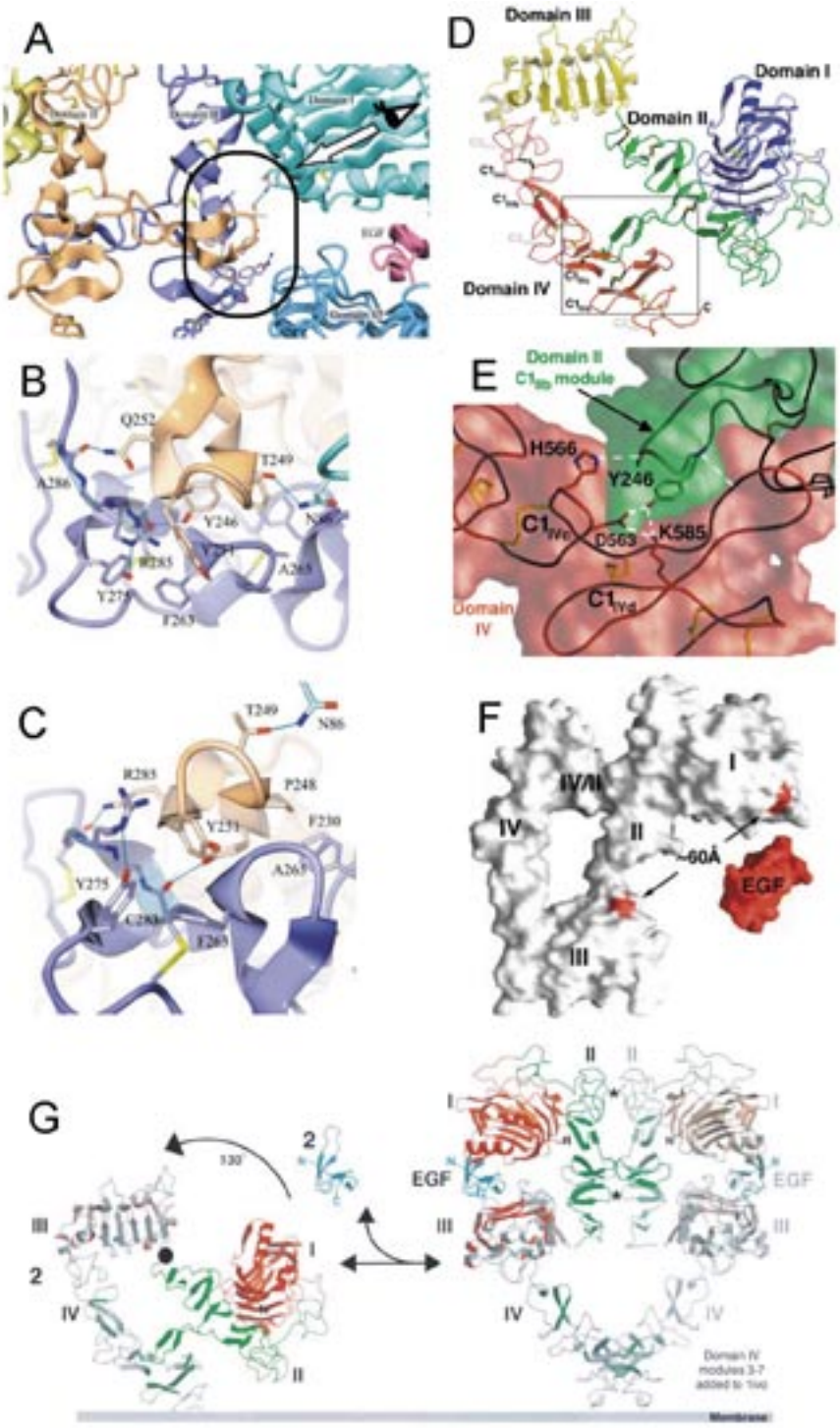
A
D
Domain III
Domain I
Domain II
Domain IV
B
Q252
T249
A286
Y246
R285
A265
Y275
F263
E
Domain II
C1ββ module
H566
Y246
C1IVc
D563
K585
Domain IV
C1IVd
C
T249
N86
R285
P248
Y251
F230
Y275
A265
F263
F
IV/II
I
IV
II
~60Å
EGF
III
G
130
2
III
I
2
IV
II
EGF
Domain IV modules 3-7 added to Tns

Figure 5. The cysteine-rich domains S1 and S2 are involved in dimerization and in the formation of the auto-inhibited conformation. A. The major dimerization interface of an ErbB1 homodimer. **B. and C.** Close-up of the binding region of the major dimerization interface from two different sites of view. A dimerization arm, formed by a 20 amino acid β-hairpin, protrudes from the S1 domain of both receptors. The tip of the arm forms a hand-like structure that interacts with the arm of the other receptor. Key residues in this interaction are Y246, P248 and Y251 in the hand-like structure which interact with F230, F263, A265, Y275 and R285 in the arm of the other receptor. Outside the dimerization arm two regions are involved in the S1-S1 interaction: D279 interacts with H280 in the other receptor while Q193, Q194 and C195 interact with P204 and S205 in the other receptor (7, 14). **D.** ErbB1 in the auto-inhibited conformation. **E.** Close-up of the S1-S2 interaction in ErbB1. Key residues in this interaction are D563, H566 and K585 (D562, H565 and K583 in ErbB3) interacting with Y246 and Y251 (Y246, F251 and Q252 in ErbB3) of the S1 domain. **F.** Surface representation of the extracellular domain of auto-inhibited ErbB3. EGF and sites in ErbB3 homologous to the sites in ErbB1 to which EGF binds are depicted in red. The distance between the two putative ligand binding sites in ErbB3 is about 60Å, indicating that a domain rearrangement is likely to occur upon ligand binding. **G.** Transition between the auto-inhibited conformation and the ligand-bound ErbB1 dimer. EGF binding is proposed to induce a 130° rotation of the receptor resulting in the exposure of the dimerization arm to allow dimerization. Figure taken from Ferguson et al. (6).

of the receptor dimer induce rotation or twist of first the juxtamembrane regions, and subsequently of the transmembrane domains, which may be necessary for the kinase domain to become appropriately positioned for phosphorylation of substrate molecules. These molecular switch models for ErbB2 dimerization explain the oncogenic properties of the ErbB2 mutant in which V664 is mutated to a Glu. V664 is part of the C-terminal motif and when this residue is mutated to a Glu, the polar side chain of E664 will be exposed to a hydrophobic lipid environment, which is energetically unfavourable. As a result, inactive ErbB2 homodimers mediated by the C-terminal motif will not be formed when V664 is mutated to E664. However, the level of ErbB2 homodimerization mediated by the N-terminal motif will be increased because of hydrogen bond formation (20), resulting in autophosphorylation of the receptors. On the other hand, a single nucleotide polymorphism in ErbB2 resulting in the V655I mutation is associated with reduced risk for mammary carcinomas (21). The molecular shift model proposes that substitution of Val by the bulkier Ile residue at this position will destabilize the formation of active ErbB2 dimers that are mediated by the N-terminal dimerization motif. Thus the transmembrane region controls dimer formation and rare mutations in this region might explain the occurrence of active ErbB2 homodimers.

4.1 The asymmetrical model of ErbB transphosphorylation

In addition to the extracellular domain, crystal structures have been determined of the intracellular region of ErbB1. The crystal structure of the

kinase domain of ErbB1 in complex with 4-anilinoquinazoline, an ErbB1-specific tyrosine kinase inhibitor (TKI), suggests that the kinase domain is always in an active conformation (22). The kinase domains of ErbB receptors differ from all other known receptor tyrosine kinases in that their activation loop (K836-848) adopts a conformation in the unphosphorylated state which is similar to that of other kinases in the phosphorylated state (22). Although these studies were performed with unliganded ErbB1, mutation of Y845 in ErbB1 into Phe does not block EGF-induced ErbB1 signaling (23), suggesting that activation of the ErbB1 kinase is independent of its phosphorylation state.

In another study, Zhang et al. (24) demonstrated that the intracellular domains of an ErbB1 homodimer can show an inactive symmetric or an active asymmetric orientation. In the symmetric inactive conformation the tyrosine kinases directly interact with each other back-to-back and thereby keep their kinase domains in an auto-inhibited orientation (Figure 6B). In the asymmetric active conformation one tyrosine kinase phosphorylates the C-terminal tail of the other receptor (Figure 6E and F). The authors assume that transphosphorylation of both ErbB1 receptors in a homodimer requires a dynamic switch in orientation of two receptor kinase domains (Figure 6E and F). In a heterodimer, however, ErbB2 and ErbB3 will prefer an orientation (Figure 6E) in which the C-terminal tail of ErbB3 is phosphorylated by the kinase of ErbB2. Evolutionary analysis showed that the amino acid sequences of ErbB1, ErbB2 and ErbB4 at the interface of the kinase that interact with the C-terminal tail of its partner receptor are highly conserved among vertebrates, while for ErbB3 many variations were found (25). This suggests that because of its inactive kinase there might be no evolutionary pressure in ErbB3 on the maintenance of residues facilitating an interaction of the kinase domain with the C-terminal domain of its partner receptor. It remains unclear, however, how ErbB2 is phosphorylated in an ErbB2-ErbB3 heterodimer by the kinase-defective ErbB3.

In addition to the extracellular domain and the transmembrane region also regions in the intracellular domain are involved in dimer formation. A sequence of three amino acid residues (LVI) at the C-terminal end of the impaired ErbB3 kinase region has been reported to be essential for transactivation by ErbB2 (26). The corresponding region in ErbB2 (VVI) was found to be necessary for ligand-independent homodimerization (27). In a crystal structure of the ErbB1 kinase domain the LVI sequence is in close contact with the C-loop of the kinase domain (22). In this interaction L955 is completely buried and therefore not available for interaction with the partner receptor or adaptor molecules. A more plausible explanation would be that the tripeptide segment mediates a rotation in the intracellular region that affects the availability of the C-terminal tail as a substrate for transphosphorylation.

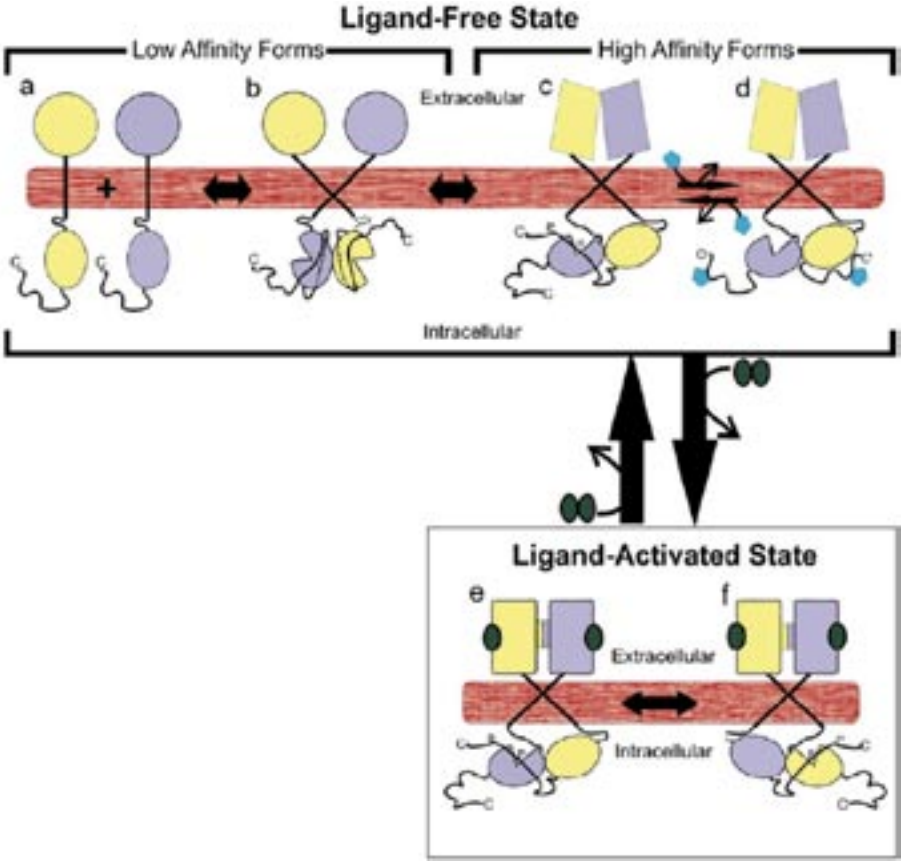

Figure 6. Asymmetric interaction of the intracellular domains in the active ErbB1 homodimer. **A and D** illustrate unoccupied ErbB1 receptors. **E and F** illustrate the ligand-activated ErbB1 dimer**. A** shows two ErbB monomers**. B** shows the low affinity form of the ErbB1 homodimer in which the kinase domains are in such orientation that they do not interact with the C-terminal intracellular domain of its partner receptor**. C and D** show high affinity forms of ErbB1 in which the intracellular domains are asymmetrically orientated**. E and F** show ligand induced ErbB1 homodimer in which the intracellular domains are asymmetrically orientated. In the asymmetrical orientations of the ErbB1 homodimers the C-terminal intracellular domain of the one receptor is bound and activated by the kinase domain of the other receptor. Figure is taken from Landau and Ben-Tal (25).

5 High and low affinity ErbB1 binding sites

Scatchard analysis of [^{125}I]-EGF binding to ErbB1 overexpressing cells has provided evidence that EGF can bind its receptor with at least two different affinities. Approximately 5% of the ErbB1 molecules bind EGF with high affinity (Kd 10-100 pM) whereas the majority of receptors (~ 95%) bind EGF with a much lower affinity (Kd 2-5 nM). Although this phenomenon has been known for decades (28), its interpretation is still a matter of debate. The observation that the anti-ErbB1 antibody 2E9 binds only low affinity receptors, indicates that low and high affinity receptors are physical entities.

Based on the crystal structure of the EGF/ErbB1 complex, it has been proposed that the monomeric tethered configuration corresponds to the majority of low affinity receptors, whereas un unliganded extended dimeric configuration corresponds to the minority of the high-affinity class of ErbB1. However, mutagenic disruption of the autoinhibitory tether did not give rise to a single receptor population that binds ligand with high affinity, and also did disruption of receptor dimerization not yield a receptor that binds EGF with only a single low affinity (29). Thus, these results demonstrate that the extended configuration of ErbB1 does not account for the high affinity EGF binding sites. Mathematical analysis by Klein et al. (30) could only explain the existence of high and low affinity binding sites by postulating an additional component to the dimeric ErbB1/EGF complex.

For measuring the inter-EGF distance and the vertical distance of EGF to the plasma membrane in the EGF/ErbB1 complex, use has been made of Fluorescence resonance energy transfer (FRET) analysis in combination with ensemble fluorescence lifetime imaging microscopy (FLIM). Using these techniques Webb et al. (31) found that high affinity ErbB1 ectodomains are orientated flat on the membrane, whereas low affinity ectodomains are oriented vertically to the plasma membrane. These studies were performed in the presence or absence of 2E9 antibodies by which selectively low affinity receptors were blocked. Moreover, they observed that the extracellular domains (ECDs) of low affinity ErbB1 dimers are orientated side-by-side, whereas ECDs of high affinity ErbB1 dimers are orientated head-to-head. Macdonald and Pike (32) explain the occurrence of high and low affinity binding sites of ErbB1 by negative cooperativity on cells that express such high levels of ErbB1 that the receptors spontaneously aggregate. The essence of their model is that the affinity of two occupied monomers for each other is, by an order of magnitude, lower than the affinity of an occupied monomer for an unoccupied monomer, implying that at high EGF concentrations, EGF should induce disassembly of receptor dimers. In conclusion, studies describing the nature of low and high affinity ErbB1 receptors are explained in very complex models. The explanation for the nature of high and low affinity ErbB1 receptors of Webb et al. (31) and Macdonald and Pike (32) have in common that the orientation of high affinity ErbB1 dimers on cells expressing high levels of ErbB1 is different from the orientation of ErbB1 monomers.

6 Mutational analysis of ligand-receptor interaction

The relative contribution of individual ligand residues and combinations of residues on ligand–receptor interaction have been studied extensively by site-directed mutagenesis, alanine-scanning mutagenesis, domain-exchange and phage display strategies. Groenen et al. (33) and Campion et al. (34) have reviewed the effect of point mutations in EGF and TGFA on their ErbB1 binding affinity. These studies showed that, in addition to the structurally important cysteines and glycines, in particular Y13/F13, L15/F15, H16, R41 and L47 are essential for high affinity receptor. Figure 2 shows the amino acid sequences of the EGF-like ligands. A phage display study in which positions 13, 15 and 16 in EGF were randomized, confirmed the importance of Y13, L15 and H16 for ErbB1 binding (35). Multiple single amino acid mutation studies demonstrated that R41 and L47 are essential for high affinity ErbB1 binding (33).

The importance of Y13 and the B-loop residues M21, Y22 and A30 for high affinity binding of EGF to ErbB1 was examined by reintroduction of these amino acids into the 13th EGF-like repeat of the Drosophila Notch protein, which has an EGF-like structure but does not bind ErbB1. Notch proteins in which combinations of these four amino acids were introduced, showed ErbB1 binding affinity similar to wild type EGF (36). Remarkably, most of the amino acids that were found to be essential for high affinity binding are involved in binding to the L2 domain of the receptor, whereas there appears more freedom in mutating the amino acids involved in L1 domain binding without loss of high affinity binding. The length of the B-loop of EGF can be shortened without loss of binding affinity, as been demonstrated by an EGF-mutant in which the B-loop is shortened to 8 amino acids (37). Also EGF with an extended B-loop of 12 amino acids following incorporation of residues from the Drosophila protein Argos, retained binding affinity for ErbB1 (38), although some loss of affinity was observed, most likely due to the absence of L26 at the correct position in the B-loop (33).

In case of NRG1β, a full alanine scanning and phage display affinity optimisation has been carried out. Alanine scanning mutagenesis showed that H2L3 in the N-terminal linear region, the complete A-loop, R31 in the B-loop, the hinge residue K45, the complete C-loop, and Q46, Y48 and M50 in the C-terminal linear region are important for high affinity ErbB3 binding (39). For high affinity ErbB4 binding the residues S1, F13, V15, N16, G42, R44, Q46, S52 and F53 appeared essential, indicating that the requirements for binding of NRG1β to ErbB3 and ErbB4 partly overlap, but are not identical. Phage display mutagenesis of the complete NRG1β EGF-like domain showed strong conservation of L3, V4, V15, N16, G17, G18, E17, V23, Q46, Y48 and V49 (40). In particular mutations in the B-loop and the M50I mutation increased the affinity for ErbB3, indicating that the wild-type sequence of NRG1β is suboptimal for ErbB3 binding. Combination of these mutations in the B-loop and the M50I mutation resulted in a neuregulin variant, designated

NRG-optimized (NRG^{opt}), which showed a 3-fold increased affinity for ErbB3. These studies also showed that the so-called omega loop (L26, S27, N28), three additional residues at the tip of the B-loop, is not essential for receptor binding of NRG1β. Shortening of its B-loop from 13 to 10 residues, as similarly present in EGF and TGFA, did not impair binding to ErbB3 and ErbB4 (41).

NRG1 and NRG2 have multiple C-terminal linear regions as a result of alternative splicing. Single amino acid exchange between residues of the C-terminal linear region of the isoforms NRG2α and NRG2β showed that exchange of K45 from NRG2α for F45 from NRG2β resulted in enhanced affinity of NRG2α and reduced affinity of NRG2β for ErbB4. These studies demonstrate that minor changes in the amino acid sequences of ErbB ligands can cause strong alterations in the ErbB binding affinity.

7 Mutational analysis of ErbB specificity

Domain-exchange studies between ligand molecules have been instrumental to study the ErbB specificity of EGF-like growth factors. Studies in which intercysteine domains were exchanged between EGF and NRG1β showed that introduction of the N-terminal linear region of NRG1β into EGF results in a chimera, designated biregulin, that binds with high affinity to not only ErbB1 but also to ErbB3 (41). We have shown that introduction of the N-terminal linear region of TGFA into EGF (chimera T1E) is also sufficient to broaden its receptor specificity to ErbB3 and ErbB4. Subsequent studies showed that introduction of the sequence H2/F3 from TGFA is sufficient to make EGF an ErbB3 and ErbB4 binding ligand. Phage display analysis revealed that EGF with an N-terminal linear region containing the sequence W2/V3/R4 shows similar high binding affinity as EGF for ErbB1, and as NRG1β for binding to ErbB3 and ErbB4. A different observation was made when a single intercysteine region of EGF was introduced into NRG1β. This left the affinity for ErbB3 and ErbB4 intact, but did not result in detectable affinity for ErbB1 (41). In contrast, introduction of a single intercysteine region of NRG1β into EGF or TGFA strongly reduced the affinity for ErbB1, without a detectable gain in affinity for ErbB3 and ErbB4 (41). These results show that relatively small changes in ligand molecules can alter their receptor specificity.

Domain-exchange studies between the extracellular regions of ErbB receptors showed that EGF is able to induce phosphorylation of a chimeric receptor in which either the L1 or the L2 domain of ErbB1 was substituted by the corresponding domain of ErbB4, but not when both domains were exchanged. In addition, NRG1β was able to induce receptor phosphorylation of a chimeric receptor in which the L1 domain of ErbB1 was exchanged for the L1 domain of ErbB4. Exchange of the L2 domain of ErbB4 by the L2 domain of ErbB1 abolished NRG1β-induced phosphorylation, which indicates that particularly sequences in the L1 domain of ErbB1 prevent binding of NRG1β to this receptor (42).

8 ErbB ligand processing

ErbB ligands are produced as transmembrane precursors which are subsequently released as soluble ligands after cleavage by cell surface proteases (10, 43). The ADAM (a disintegrin and metalloprotease) family members ADAM9, ADAM10, ADAM12 and ADAM17 (a.k.a. TACE) and MMP (matrix metalloprotease) proteases are largely responsible for shedding of membrane-bound ErbB ligands (44). The production of soluble ErbB ligands through ectodomain shedding can occur in response to the activation of G-protein coupled receptors (GPCR) (45). Stimulation of GPCR leads to the activation of metalloproteases that induce cleavage and release of ligand, which subsequently activate ErbB receptors on its cell surface, thus leading to the rapid ErbB phosphorylation (46). While *in vitro* membrane-anchored proTGFA and proHB-EGF are biologically active and can interact with the ErbB receptors on adjacent cells, ectodomain shedding of the ligands appears essential for ErbB activation *in vivo*. ADAM10 is the main sheddase for EGF and BTC. In ADAM17-deficient cells no shedding of TGFA, HB-EGF and AREG is observed (47-49), and consequently ADAM17 knockout mice die prenatally with a phenotype that resembles that of HB-EGF and ErbB1 knockouts. Although there is redundancy among the ADAM family members, ADAM9, ADAM10 and ADAM12, which are also known to be involved in HB-EGF shedding, cannot restore the effect of ADAM17 depletion which controls shedding of HB-EGF during heart development (50).

Similar to TGFA and HB-EGF, NRG is cleaved by ADAM17. In case of the Ig-NRGs (Type I and II) this results in release from the plasma membrane. In case of the cysteine-rich domain (CRD)-NRGs (Type III) the ligand remains bound to the membrane, since they have an additional transmembrane domain. The CRD-NRGs are biologically active as well as the transmembrane Ig-NRG precursor (51). CRD-NRG knock-out mice die shortly after birth because they cannot breath due to the absence on functional neuromuscular synapses (52). Furthermore, the number of skeletal muscle cells and Schwann cells are severely reduced in CRD-NRG knock-out mice compared to wild-type mice. Ig-NRG knock-out mice, with normal levels of CRD-NRG, show a normal development of Schwann cell precursors, but die at E10.5 due to defects in cardiac, cranial sensory and sympathetic neuron development similar to pan-NRG knock-out mice (53). In contrast, CRD-NRG knock-out mice have a normal development of the heart (52).

Overexpression of ADAMs and MMPs, on the other hand, results in extensive ErbB ligand shedding, which can lead to pathological conditions such as myocardial hypertrophy (54) and cancer (55, 56). Therefore, ADAMs and MMPs are interesting drug targets for the treatment of cardiac disease and cancer.

9 ErbB-induced signaling pathways

The combination of four receptors and multiple ligands allows for a high degree of signal diversification, with multiple regulatory levels controlling the cellular outcome. The biological responses will depend on the identity of the ligand, the nature of the receptor dimer formed, and the identity, magnitude and duration of the induced intracellular signals. Upon ligand-induced receptor homo- and hetero-dimerization the tyrosine kinase domains of both receptors are activated which transphosphorylate tyrosine residues in the C-terminal region of their intracellular domain (57, 58). These phosphorylated tyrosines form binding sites for SH2 and PTB domain containing signaling and adaptor molecules (reviewed in (59)). The 5-8 amino acids surrounding the phosphorylated tyrosine determine the specificity for binding of SH2 and PTB containing proteins. Each ErbB receptor contains a unique set of tyrosine residues which bind a distinct subset of signaling molecules (60) (Figure 7).

The three main signal transduction pathways activated by the ErbB family members are the RAS/mitogen-activated protein kinase (RAS-MAPK) signaling cascade, the phosphatidylinositol 3'-kinase/protein kinase B (PI3K-PKB/Akt) pathway and the phospholipase C-gamma/protein kinase C (PLCγ-PKC) pathway (reviewed in (61)). The RAS-MAPK cascade is initiated by the

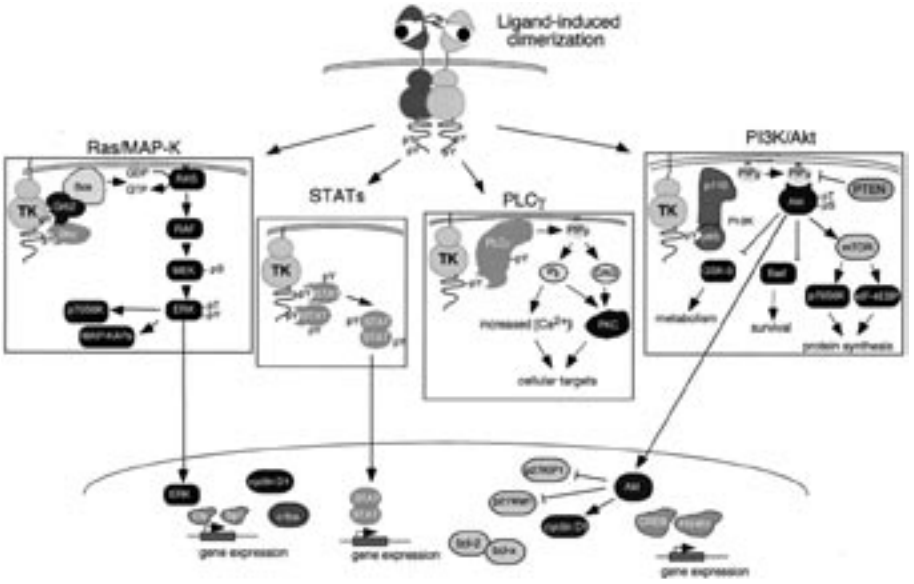

Figure 7. ErbB-induced signaling pathways. The four main signal transduction cascades activated upon ErbB activation are the RAS-MAPK, PI3K-Akt, PLCγ-PKC, and the STAT pathways. Collectively, these pathways culminate in cellular proliferation, migration, and survival. Figure taken from Marmor et al. (61).

binding of Grb2 to phosphorylated tyrosines, either directly via its SH2 domain or indirectly through PTB domain-mediated binding of the adaptor molecule Shc. Grb2 recruits Sos, which uploads Ras with GTP. Ras activates the serine/threonine kinase Raf, which results subsequently in the phosphorylation and activation of MEK1/2 (MAPKK) and Erk1/2 (MAPK). Erk1/2 phosphorylates multiple cytoplasmic and cytoskeletal proteins including MAPK-activated protein kinases and the ribosomal p70-S6 kinase. After translocation to the nucleus MAPK activates the transcription factors Sp1, E2F, Elk-1 and AP1. Sp1 and AP1 are responsible for the upregulation of cyclin D1, which activates the cyclin-dependent kinases CDK4 and CDK6, and thereby promotes cells to progress into the S-phase.

ErbB-induced activation of the PI3K-PKB/Akt pathway starts by the SH2-mediated recruitment of the p85 protein (reviewed in (62)), which is the regulatory subunit of the p110 catalytic subunit of PI3K. This kinase is able to phosphorylate membrane-bound PIP2 to PIP3. PIP3 subsequently recruits PKB/Akt through its PH domain to the plasma membrane, where it is subsequently activated by phosphorylation through PDK1 and an as yet unknown kinase. Activated Akt promotes cell survival by phosphorylation of the pro-apoptotic molecule Bad, which as a result is no longer able to dimerize with Bcl-2 and Bcl-X. Akt also promotes cell cycle progression by downregulation of the cyclin-dependent kinase inhibitor $P27^{KIP1}$. Furthermore, Akt negatively regulates the activity of Raf and GSK-3 kinase as well as of the cell cycle regulatory transcription factor FKHR, while it promotes initiation of protein translation through mTOR and the ribosomal p70-S6 kinase.

PLCγ is recruited to the ErbB receptor either directly by binding through its SH2 domain or indirectly through binding with its PH domain to PIP2 (*61, 62*). Activation of PLCγ by the ErbB kinase of by other cellular kinases results in the hydrolysis of PIP2, thus generating the second messengers DAG and IP3. Binding of IP3 to the IP3 receptors on the endoplasmic reticulum leads to Ca^{2+} influx from the endoplasmic reticulum (ER) to the cytoplasm. Increased cytoplasmic Ca^{2+} levels lead to the activation of calcium/calmodulin-dependent protein kinases and phosphatases. Together with DAG high cytoplasmic Ca^{2+} concentrations will result in activation of protein kinase C (PKC), a serine/threonine kinase that among others can activate Raf and downstream pathways.

Another pathway that can be activated by ErbB receptors is c-Src. This oncogen product is able to phosphorylate additional tyrosine residues in ErbB1, as well as in cytoskeletal and endocytic proteins, and in addition is able to activate the oncogenic transcription factor c-Myc (63). Furthermore, ErbB1 is able to directly activate the nuclear transcription factors STAT1, STAT3 and STAT5 (64). Upon tyrosine phosphorylation the cytoplasmic STAT proteins dimerize, translocate to the nucleus and activate transcription of genes, which are critical for proliferation.

A more recently identified adaptor protein that binds tyrosine phosphorylated ErbB1 is Gab1. Together with the tyrosine phosphatase Shp2

it forms an ErbB1 specific interaction between the RAS/MAPK cascade and the PI3K/Akt pathway (65). In contrast to ErbB3, ErbB1 cannot directly associate with the p85 subunit of PI3K. ErbB1-mediated PI3K activation involves the adaptor proteins Grb2 and Gab1 (66). While Grb2 directly associates with ErbB1, Gab1 binds ErbB1 indirectly via Grb2, after which it is able to bind the p85 subunit of PI3K. The ErbB1-activated adaptor protein Shp2 controls the ErbB1-mediated PI3K activation by dephosphorylating the p85 binding sites on Gab1, thereby terminating the Gab1-PI3K positive feedback loop.

Despite sharing some pathways, each ErbB receptor interacts with a different set of signaling molecules. The RAS-MAPK pathway and the PI3K-Akt pathway are activated by all four ErbB receptors, but not to the same extent. The potency and kinetics of the PI3K-Akt pathway, however, is quite different. Since ErbB3 has six docking sites for p85, the ErbB2-ErbB3 heterodimer is a very strong activator of the PI3K-Akt pathway. ErbB4 can also directly interact with p85, but only through one docking site, while ErbB1 depends on Gab1 for activation the PI3K-Akt pathway. ErbB3 is the only ErbB receptor that does not interact with Cbl, while ErbB1, ErbB2 and ErbB4 have functional Cbl binding sites (67). Furthermore, ErbB3 does not have sites for binding to the adaptor protein Grb2, but can interact with the adaptor proteins Shc and Grb7 (63).

As a result of alternative splicing there are four ErbB4 isoforms, which differ in their signaling pathways. The JM-a isoform can be cleaved by TACE and γ-secretase at the extracellular juxtamembrane region, resulting in the formation of a soluble extracellular fragment and a transmembrane fragment. Upon further cleavage this leads to the formation of an intracellular fragment (ErbB4 s80) that can translocate to the nucleus where it binds nuclear proteins including transcription factors. In contrast, the JM-b isoform cannot be cleaved in his way and gives rise to a full length transmembrane receptor. For both the JM-a and JM-b isoform there are two further subforms, Cyt-1 and Cyt-2, which differ in their intracellular domain. In contrast to Cyt-1, the Cyt-2 subforms lack the PI3K binding motif and are therefore unable to activate the PI3K/Akt pathway. The JM-b mediated form of signal transduction, relying on activation of tyrosine kinase activity, is referred to as canonical ErbB4 signaling, whereas the JM-a mediated form, relying on direct binding of nuclear proteins, is referred to as non-canonical ErbB4 signaling. It has been established that non-canonical ErbB4 signaling regulates the activity of at least the STAT5A, Eto2, Mdm2 and YAP nuclear proteins. It has been established that NRG1β and BTC are able to induce different second messengers upon binding to ErbB4 receptors. Whether this implies that ligands may preferentially induce canonical or non-canonical ErbB4 signaling, is currently unknown (10).

There is increasing evidence that upon activation also other ErbB receptors can be translocated to the nucleus where they are directly involved in transcriptional activation (68-70). However, the significance of ErbB nuclear translocation in signal transduction is still a subject of debate. Nuclear ErbB1

has been shown to recognize an AT-rich response sequence (ATRS) in the promoter of the cyclin D1 and iNOS gene (68, 69). Nuclear ErbB2 appears associated with the cyclo-oxygenase (COX-2) promoter where it interacts with so called HER-2-associated DNA sequences (HAS) (71). The presence of ErbB3 has been reported in the nucleus of mammary epithelial cells, but a functional role for a nuclear ErbB3 has not been attributed yet (70).

9.1 Negative regulation of ErbB signaling

Upon ligand binding activated ErbB1 is rapidly removed from the cell surface by internalization through clathrin-coated pits (reviewed in (72)). The phosphorylated receptor recruits the adaptor protein c-Cbl, a ubiquitin ligase which is responsible for ubiquitination of the receptor (73). Subsequently, the endocytic adaptor molecule Eps15 recruits the ubiquitinated receptor to the clathrin-coated pits by targeting AP-2 to the plasma membrane (74, 75). AP-2 complexes on the membrane then drive the assembly of clathrin-coated vesicles by creating a coated pit. The release of the clathrin-coated vesicle (early endosome) from the plasma membrane involves the GTPase dynamin (reviewed in (76)). Fusion of the early endosome with other internal vesicles forms the sorting endosome from which unoccupied receptors can recycle back to the plasma membrane (reviewed in (77)). Receptors that are still bound by their ligand remain in the endosome, which matures to a late endosome (multi-vesicular body or MVB). During endosomal maturation the pH of the lumen decreases due to the action of the vacuolar H^+ATPases. Before entering the multi-vesicular bodies ligand-bound ErbB receptors are still capable of signaling (78). Once in the MVB the cytoplasmic tail is sequestred from the cytoplasm which prevents its interaction with cytoplasmic signaling molecules (79). After fusion with pre-existing lysosomes, both the receptor and the ligand are finally degraded by the lysosomal proteases (80). Upon activation of ErbB1 by EGF the EGF-ErbB1 complex is internalized via clatherin-coated vesicles and continues to signal in the endosomes until both are degraded in the lysosomes. TGFA on the other hand also induces receptor internalization but dissociates from the receptor due to the low pH in the endosomes, resulting in receptor recycling to the cell surface where it can be reactivated.

UBPY accelerates ErbB1 degradation by deubiquitinating the receptor on endosomes (81, 82). Also Sprouty has been identified as a protein that affects ErbB1 ubiquitination. Originally, Sprouty has been identified as an inhibitor of Drosophila EGF receptor signaling (83). Its mammalian homologues have been shown to inhibit ErbB1 signaling and to prevent MAPK phosphorylation (reviewed in (83)). Activation of ErbB1 induces enhanced transcription of the Sprouty-2 gene and phosphorylation of the Sprouty protein. Phosphorylated Sprouty binds c-Cbl, which on the one hand prevents ErbB1 ubiquitination, but on the other hand targets Sprouty for lysosomal degradation (84). Recent publications show that Sprouty-2 associates with

the multi-adaptor protein CIN85 and couples Cbl to CIN85 in order to inhibit ErbB1 ubiquitination (85). CIN85 probably functions as a scaffold molecule that binds to numerous endocytic accessory proteins and thereby controls trafficking of ErbB1 along endocytic and recycling pathways.

Caveolae are specialized forms of lipid rafts that are marked by the presence of the protein Caveolin-1 (86). With the exception of ErbB3, the ErbB family members are preferentially located in caveolae, while upon ligand binding ErbB1, ErbB2 and ErbB4 migrate out of the caveolae to the bulk of the plasma membrane. The Caveolin-1 gene has been described as a tumor suppressor gene since down regulation of Caveolin1 is sufficient for MAPK activation and cell transformation (87). The extracellular juxtamembrane region of ErbB1 contains a sequence that targets the receptor to the caveolae/rafts (88). Interaction of ErbBs with Caveolin-1 results in inhibition of their tyrosine kinase activity (89). Since caveolae are enriched in signaling molecules like RAS and Src, and Caveolin 1 inhibits receptor tyrosine kinase activity, caveolae might regulate the activity of early ErbB signaling in these preassembled complexes.

Decorin, a small leucine-rich proteoglycan, has been described as a negative regulator of ErbB1 signaling. Decorin causes a slow but sustained internalization of unliganded ErbB1 via caveolae-mediated endocytosis (90). These so-called caveosomes are non-clathrin coated vesicles that similar to clathrin-coated endosomes fuse with the late endosome, resulting in lysosomal degradation of the receptor. Unlike unliganded receptors in the early endosome, the receptors in the caveosomes cannot recycle to the plasma membrane. Decorin directly interacts with the L2 domain of ErbB1 (91), and since the decorin binding site partially overlaps with the ligand binding site, this protein may decrease the available number of ligand binding sites on the cell surface. In addition to clathrin-mediated and caveolin-mediated endocytosis, clathrin- and caveosome-independent endocytosis of ubiquitinated ErbB1 has been reported to occur in the lipid rafts. This process of so-called raft-endocytosis occurs particularly at high ligand concentrations (92).

Ectodomain shedding of ErbBs by secretase enzymes and TACE may negatively regulate ErbB signaling. The resulting soluble extracellular domains of ErbB3 and ErbB4 have been shown to act as scavengers of NRGs, and thereby prevent their binding to the full length membrane-bound ErbB3 and ErbB4 (93). The recombinant extracellular domain (ECD) of ErbB1 (amino acid 30-501) also acts as a scavenger of ErbB1 ligands, but there is no evidence that ECD of ErbB1 occurs naturally (94). A soluble extracellular domain of ErbB2 has been identified, which is not formed by proteolytic cleavage, however, but instead results from alternative splicing of the ErbB2 transcript (95). This splicing product, known as Herstatin, consists of the extracellular domain of ErbB2 linked to a C-terminal proline-rich domain. Herstatin prevents the formation of active heterodimers by binding with its L1 domain to liganded ErbB receptors or unliganded ErbB receptors in

the extended conformation (96). Ectodomain shedding of ErbB2, however, results in the formation of a constitutively active p95 fragment, as observed in breast cancer cells. Prevention of ErbB2 ectodomain shedding by Herceptin is therefore one of the mechanisms by which this monoclonal antibody is successful in the treatment of breast cancer.

An additional negative feedback loop in ErbB signaling is provided by the products of the three LRIG genes. The best characterized member, LIG-1, is upregulated upon ligand-induced ErbB activation (97). Targeted disruption of the LIG-1 gene causes psoriasis-like epidermal hyperplasias (98). Since ErbB1 is involved in skin development, the LIG-1-null phenotype might be linked to ErbB1 signaling. LIG-1 directly interacts with all four members of the ErbB family. The extracellular domain of the LIG-1 proteins consists of fifteen leucine-rich regions (LRR) and three immunoglobulin-like (Ig) domains, which both are sufficient for ErbB1 binding (99). LIG-1 binds ErbB1 and stimulates ubiquitination of both LIG-1 itself and of ErbB1 by recruiting Cbl, thereby reducing the half-life of the receptor. Although LIG-1 expression is upregulated by ErbB activation, the LIG-1-targeted receptors require receptor activation, with the consequence that LIG-1 reduces the number of ligand binding sites on the cell surface. The LRIG genes have been reported as homologues of the Drosophila EGF receptor antagonist Kekkon which also contains LRR and Ig domains. However, the working mechanism of Kekkon is different, since this protein interferes on the level of ligand binding and receptor dimerization (100).

9.2 Juxtacrine signaling by ErbB ligands

Membrane-ancored precursors of TGFA, AREG, HB-EGF and NRG1 are able to bind and activate ErbB receptors in a juxtacrine fashion. In contrast to the soluble ligands forms, these non-diffusible ligands can only transmit their signals to neighbouring cells, which ensures signaling in a proper temporal and spatial context. Juxtacrine activation of ErbB1 by AREG has been shown to play a role in trophoblast growth in the placenta (101). Membrane-bound ligands may have additional functions besides activating ErbB receptors on neighbouring cells. The transmembrane form of TGFA interacts with CD9 (102), a tetraspanning transmembrane molecule that binds various integrines (103), which suggests that the transmembrane form of TGFA may in addition be involved in cell adhesion. This hypothesis is strengthened by the observation that ErbB1, together with E-cadherin and α- and β-catenin, is localized at the zona adherens in polarized cells (104). Increased CD9 expression inhibits TGFA release by metalloprotease activity (102) and inhibits motility and metastasis in carcinoma cell lines (105, 106), while decreased CD9 expression is correlated with poor prognosis in breast cancer. CD9 co-precipitates with AREG and HB-EGF in keratinocytes resulting in increased juxtacrine ErbB activation (107).

Transmembrane isoforms of NRG1 have been reported to mediate bidirectional signaling in neurons (108). In the model of back signaling NRG1 is proteolytically cleaved upon ErbB receptor binding. Consequently the intracellular domain is released and is translocated to the nucleus where it represses expression of several regulators of apoptosis. In addition the intracellular region of NRG1 interacts with LIMK1 (109), a cytoplasmic protein kinase implicated in development of visuospatial cognition. LIMK1 shuttles between the nucleus and the cytoplasm and is involved in transcription regulation, but the significance of LIMK1 in NRG1 back signaling remains to be demonstrated (110).

10 ErbB function in development and differentiation

The expression of the four ErbB receptors and the eleven ligands differs spatially and temporally during development. Table I gives an overview of the site of expression and known *in vivo* function of all ErbB ligands. Knock-out mice have shown crucial roles of the ErbB family members and their ligands in development of the cardiovascular system, nervous system, epithelium and mammary gland. Table II gives an overview of the results of these knock-out studies. The specific developmental role of the various NRG isoforms is described in a separate section.

10.1 Cardiovascular system

The importance of ErbB2, ErbB3, ErbB4 and their ligand NRG1 in the development of the cardiovascular system has been demonstrated by the use of knock-out mice lacking these genes. Mice lacking ErbB2, ErbB4 or NRG1 die at embryonic day 10 due to aborted development of myocardial trabeculae in the heart ventricle (111-113). As a result this mutant has a heart with irregular beat, an enlarged common ventricle and a reduced blood flow. Mice lacking ErbB3 survive until embryonic day 13 and exhibit cardiac cushion abnormalities leading to blood reflux through defective valves (114). HB-EGF knock-out mice and mice lacking ErbB1 and TACE (ADAM17) have defective valvogenesis. This suggests that cardiac valvogenesis is dependent on ErbB1 activation by TACE-derived soluble HB-EGF.

10.2 Nervous system

NRG1 and its receptors are very important for neuronal development. NRG1 is produced by neurons and regulates glial cell proliferation, axon myelination, neuronal migration, axon guidance and synapse formation. During early brain development NRG1, together with ErbB4, provides

Table I. Function and sites of expression of ErbB ligands

Ligand	Site of expression	Function	References
EGF	Saliva, urine, and glands of small intestine	Wound healing; Premature eye opening in newborn eyes	(60)
TGFA	Tumor epithelial cells, macrophages, brain and eye	Wound healing; rhythmic expression in the suprachiasmatic nucleus reversibly inhibits locomotor activity and sleep	(123,124,200)
BTC	Pancreatic β-cells Milk	Differentiation of β-cells in insulin-secreting cells; Suggested role in development of the gastrointestinal tract of the newborn; Indirect role in angiogenesis and pigmentation of the skin	(201) (202)
HB-EGF	Macrophages, lung, brain and heart skeletal	Cell migration, chemotaxis and proliferation	(47,104,203)
AREG	Breast	Ductal outgrowth during puberty and pregnancy;	(204)
	Ovary	Ovulation and luteinization in the human ovary.	(205)
EREG	Intestine, ovary vascular smooth muscle cells (VSMC)	Wound healing; Ovulation and luteinization in the human ovary; Paracrine differentiation factor for VSMC	(206) (205) (207)
EPG	Keratinocytes Adult testis, heart, liver.	Unknown	(208)
NRG1α	Neurons, astrocytes, fibroblasts, keratinocytes, skeletal muscle, neuromuscular junction, breast liver, lung.	Maintenance factor in myocardial epithelium, nervous system and breast development	(209)
NRG1β	Heart, nervous system, lung, brain and breast	Synaptic modulator at the neuromuscular junction; Branching morphogenesis in the lung; Neuron migration in the brain; Development of the foetal heart	(10,108,209-211)
NRG2	Brain Embryonic heart/atrium	Reproduction	(212)
NRG3	Forebrain; Future mammary region	Neuroblast migration; Permissive role in initiation of mammary gland development	(213) (214)
NRG4	Pancreas Uterus	Islet morphogenesis and beta-cell differentiation; Embryo-uterine cross-talk during implantation	(215) (215,216)

patterning information for the proper migration of neural crest cells. For the formation of the projection of the thalamus to the cortex the axons are guided by GABAergic interneurons that express type III NRG1β (115). The thalamo-cortical axon conveys the sensory and motor input to the cerebral cortex. The appropriate direction of ErbB4-expressing thalamo-cortical axon migration, from the ganglionic eminence towards the developing cortex, is navigated by a gradient of soluble Type I and Type II NRG1β which serve as a chemoattractant (116). *In vivo*, loss of NRG1/ErbB4 signaling causes an alteration in the migration of cortical interneurons and a reduction in the number of GABAergic interneurons in the postnatal cortex (117). NRG1 regulates synaptic plasticity by activation ErbB4 on the presynaptic GABAergic interneurons, thereby enhancing activation-dependent GABA release (118). A role for ErbB1 in neurogenesis is indicated by the observation that mice lacking ErbB1 develop strain-dependent progressive neurodegeneration (119).

10.3 Neuregulin and ErbB4 involvement in schizophrenia

Schizophrenia is a disabling neurophysiological disorder that affects 0.5%-1% of the world's population and is characterized by abnormalities in the perception of reality. Genetic linkage studies in multiple populations from six different countries have identified more than 80 single nucleotide polymorphisms (SNPs) in the human NRG1 gene (118) and 18 in the human ErbB4 gene (120, 121). Most of these SNPs are localized in the 5' region of the NRG1β gene and there is increasing evidence that these SNPs may regulate NRG1 expression (121-123). Brain samples of schizophrenia patients show increased levels of Type I NRG1β (122). The upregulated expression of the CYT1 isoform of ErbB4 in schizophrenia patients suggests an important role of the PI3K-Akt pathway. Moreover, risk factors for schizophrenia, such as corticosteroids and cannabis, suppress the activity of the PI3K-Akt pathway (124). The combination of increased levels of NRG and ErbB4 transcripts in brain samples of schizophrenia patients attribute to the so-called "Gain-of-function and hypoglutamatergic function in schizophrenia hypothesis (118) This implies that increased ErbB4 signaling stimulates GABA transmission, which reduces the firing rate of glutamate-dependent neurons. Hypofunction of the glutamatergic-pathways has been found in the brains of patients with schizophrenia (125).

10.4 Epithelial development

ErbB1 knock-out mice differ in their phenotype, depending on their genetic background. Such mice either die at either mid-gastrulation (SV129 strain), at birth (C57BL/6 strain) or on postnatal day 20 (CD1, C3H and MF1

Table II. Knockout studies of ErbB ligands and their receptors

Gene	Survival	Phenotype	References
ErbB1	Die around gastrulation / Die short after birth	Immature lungs, defects in the epidermis, placenta and brain	(116,213-216)
ErbB2	Die at embryonic day 10	Pre- and postsynaptic defects of developing neuromuscular junction	(108,109,206,216-218)
	Targeted disruption ErbB2 in mammary	Impaired ductal outgrowth	(219)
ErbB3	E13.5	Cardiac cushion abnormalities leading to blood reflux through defective valves; Lack of mature neuromuscular junction; The cranial ganglia defects, along with a dramatic reduction in Schwann cells, enteric ganglia and adrenal chromaffin cells, suggests a generalized effect on the neural crest; Anomalous development of the stomach and pancreas.	(109,206,220)
ErbB4	E10.5	Misprojections of cranial sensory ganglion efferent axons; Aborted development of myocardial trabeculae in the heart ventricle. Alterations in innervation of the hindbrain in the CNS	(107,221)
EGF	Viable	No overt defects	(200)
TGFA	Viable	Hair follicle and eye abnormalities	(121,122)
NRG1	E10.5	Heart malformations Schwann cell precursors and cranial ganglia fail to develop normally	(106,109,206)
NRG2	Viable	Early growth retardation and reduced reproductive capacity	(208)
BTC	Viable	No overt defects	(46)
AREG	Viable	Ductal outgrowth effects in the mammary gland	(200)
EPR	Viable	Redix edresponse to intestinal injury	(46,202,202)
HB-EGF	>50% died in the first postnatal week	Heart failure with enlarged ventricular chambers and enlarged cardiac valves Poorly differentiated lungs	(46,202)
EGF/ TGFA/ AREG triple null	Viable	Poorly organized alveoli in the mammary gland and decreased expression of milk proteins; Redix edresponse to intestinal injury	(200) (222)
HB-EGF/BTC	>50% died in the first postnatal week	Heart failure with enlarged ventricular chambers and enlarged cardiac valves; Poorly differentiated lungs	(46)

E=represents the number of embryonic days

strains). Mice lacking ErbB1 exhibit strain-dependent phenotypes ranging from placental to postnatal skin and lung defects. Poorly differentiated lungs are also observed in HB-EGF deficient mice (50). TGFA plays a role in determining skin architecture and hair development, as indicated by the observation that TGFA (-/-) mice show a misalignment of the hair follicles, known as waviness of the coat. Although these mice are healthy and fertile, some older mice suffer from corneal inflammations as well as from lens and retinal defects (126, 127).

10.5 Mammary gland

The development of the mammary gland is very complex. AREG, BTC, HB-EGF, EPR, EGF, NRG1 and TGFA each have a unique temporal expression pattern during mammary development, maturation and involution (128). In addition there is a dynamic expression of all four ErbB receptors during postnatal development. During ductal morphogenesis ErbB1 and ErbB2 colocalize, but they are localized differently in the mature gland. ErbB1 and ErbB2 are preferentially expressed in both lactating ducts and aveoli, while ErbB3 and ErbB4 expression is restricted to the aveoli. During pregnancy and lactation ErbB3 and ErbB4 expression is upregulated, but after involution ErbB4 is absent in the mammary gland (129). A switch from ErbB3 to ErbB4 expression has been observed in the developing mammary gland, suggesting that the two receptors play different roles in mammary morphogenesis. ErbB1 and ErbB2 are highly expressed at puberty, suggesting a role of these receptors in proliferation at this stage of development, while both are expressed to a minor extent in late-stage pregnancy and lactation (130). To establish the role of ErbB4 in the development of the mammary gland, ErbB4 knock-out mice have been developed that re-express ErbB4 under the regulatory control of the cardiac α-myosin heavy chain promoter (131). These cardiac-rescued ErbB4 knock-out mice are viable, but their mammary lobular alveoli fail to differentiate correctly and lactation is defective.

10.6 Developmental role of NRG isoforms

A mentioned earlier at least 15 different NRG1 isoforms are produced from the NRG1 gene as a result of alternative splicing and alternative promoter use (reviewed in (10)). The biological significance of some of the NRG1 isoforms has been studied using knockout mice. Mice that form NRG1 type III, but lack type I and type II, die at E10.5 because of defects in cardiac development and show defects in cranial sensory neuron and sympathetic development, similar to mice in which the NRG1 gene has been completely knocked out (132). Interestingly, these mice have normal Schwann cell production (53), indicating that Type III NRG1 is involved in formation of such

cells. Type III knock-out mice do not show heart defects, indicating that type III NRG1 is not involved in heart development (52). However, these type III knock-out mice die immediately after birth, because they have no functional neuromuscular synapses and cannot breath. They also show a reduction in the number of spinal motor, sensory neurons and Schwann cells. Mice that cannot form the NRG1α isoform do not have abnormalities in heart or neuron development, but in stead show defects in breast development (133), indicating that NRG1α is essential for the development of heart and neuron tissues and NRG1β for breast development.

11 ErbB receptors and cancer

11.1 Overexpression of ErbB1 and ErbB2 in tumors

Overexpression of ErbB1 as a result of gene amplification or increased transcription has been reported in lung, pancreas and breast tumors (134) and is associated with poor prognosis. Moreover, 80% of the head and neck tumors show overexpression of ErbB1 (135). ErbB2 overexpression has been reported for breast, lung, pancreas, colon and ovarian cancer and also correlates with poor patient survival (4, 136). ErbB3 is overexpressed in Non-Small Cell Lung Carcinoma (NSCLC), Head and Neck Squamous Cell Carcinoma (HNSCC) and primary breast carcinomas, which negatively correlates with tumor survival upon treatment with ErbB1 or ErbB2 directed drugs (137, 138). In addition, many tumors are able to produce ErbB ligands themselves, resulting is autocrine receptor activation and constitutively high autophosphorylation levels (139-141).

In vitro, overexpression of ErbB1 in NIH3T3 cells results in a transformed phenotype in the presence of ligand (142). In contrast, overexpression of ErbB2 alone induces only small colonies of NIH3T3 cells in soft agar, indicating that ErbB2 alone is unable to induce anchorage independent cell growth, an *in vitro* characteristic of cellular transformation. ErbB2-induced transformation of these cells depends on heterodimerization with ErbB3, which is triggered by endogenously produced neuregulin (143). In a variety of human breast tumor cells, transcriptional downregulation of ErbB3 expression has been shown to impair proliferation, indicating that ErbB2 overexpression alone is not sufficient to drive breast tumor cell outgrowth.

Furthermore, in breast tumors (144) and in gliomas (145) ErbB1 gene amplification is often accompanied with structural rearrangements of the gene. The most frequently occurring ErbB1 variant in glioblastomas is the constitutively active EGFRvIII. This variant has a deletion of exons 2-7, resulting in a lack of the L1 domain and part of the S1 major dimerization interface. It has been speculated that the lack of the S1 domain abolishes the formation of the auto-inhibited tethered conformation (6), resulting in constitutive activation of this ErbB1 variant (146). Since EGFRvIII lacks the

major dimerization interface it is unable to dimerize (147). This observation demonstrates that dimerisation is not essential for activation of the kinase domain. In a subgroup of patients with NSCLC activating mutations have been found in the ErbB1 tyrosine kinase domain. Interestingly, these patients have a higher probability of responding to ErbB1 tyrosine kinase inhibitors than patients with wild-type ErbB1 (148-150). Although mutations in the kinase domain of ErbB2 have been found in NSCLC patients (151) overexpression appears the principal mechanism by which ErbB2 mediates breast cancer.

11.2 Role of ErbB3 in tumor progression

While many publications focus on overexpression of ErbB1 and ErbB2 in tumors, much less is known in about the role of ErbB3 and ErbB4 in tumorigenesis. The low frequency of ErbB3 and ErbB4 overexpression in carcinomas agrees with the observation that ErbB3 alone is insufficient for NRG1-mediated transformation of NIH3T3 cells (143). Autocrine activation of the ErbB2-ErbB3 heterodimer by NRG1α has been observed in NSLCSs and could be blocked by antibodies that prevent ligand binding to ErbB3 (152). ErbB2-ErbB3 heterodimers contain multiple binding sites for the p85 subunit of PI3K, and as a consequence activation of these receptors induces a strong anti-apoptotic signal for e.g. breast tumors.

11.3 Role of ErbB4 in tumor progression

Reports on the role of ErbB4 in tumorigenesis are controversial. ErbB4 has weak tumor suppressor activity in breast cancer, while the presence of ErbB4 in lung cancer has been associated with increased proliferation. A possible explanation for the controversial role of ErbB4 tumor progression might result from the different isoforms of this receptor. In both normal and malignant breast tissue, both CYT-isotypes are expressed at equal levels, whereas in breast cancer cells only the cleavable JM-a isoforms are present (153). Overexpression of the JM-a CYT2 isoform enhances proliferation of breast cancer cells while overexpression of the corresponding JM-b isoform does not (154). Interestingly, expression of the JM-a isoform is much higher in estrogen receptor positive cells than in estrogen receptor negative cells (153). Increasing evidence suggests that the intracellular fragment of ErbB4 may be a direct coactivator of the estrogen receptor (155).

12 ErbB targeting in anti-cancer therapy

Overexpression of ErbB1 and ErbB2 has been associated with a poor clinical outcome in patients suffering from epithelial cancers. Therefore ErbB1

and ErbB2 are important targets for cancer therapy. Several antibodies directed against the extracellular domain of ErbB receptors as well as inhibitors that target the kinase domain of the receptors (TKIs) are approved for clinical use or in advanced clinical trials. Treatment of tumor cells with ErbB inactivating antibodies or TKIs rapidly downregulates PI3K-Akt, MAPK, Src and STAT signaling and, as a consequence, blocks the proliferation of tumor cell lines and xenografts in nude mice (156-158).

Herceptin/trastuzumab is one of the best characterized antibodies for anti-cancer treatment and is currently used in the clinic for treatment of a.o. estrogen-insensitive breast cancer. Herceptin is the humanized form of the mouse monoclonal 4D5. *In vitro,* Herceptin downregulates cell surface ErbB2 by inducing receptor endocytosis and degradation (159) and inhibits cell cycle progression by inducing the formation of p27Kip1/Cdk2 complexes (160-162). Furthermore Herceptin inhibits the basal and activated ErbB2 ectodomain cleavage in breast cancer cells, thereby preventing the formation of a constitutively active p95 fragment (163). Crystallographic data showed that Herceptin binds to the juxtamembrane region of ErbB2 thereby blocking the cleavage site (164) (Figure 8). *In vivo*, Herceptin acts in addition by inducing antibody-dependent cell-mediated cytotoxicity (ADCC) against ErbB2 overexpressing tumor cells (162). Furthermore, Herceptin down-regulates the production of vascular endothelial growth factor production by tumor cells both *in vitro* and *in vivo* (165). In a study in which the efficiency of Herceptin on ErbB2 overexpressing breast cancer cells was determined, 26% of the patients showed reduction of tumor size. Of all patients 6% showed a total disappearance of radiographically apparent tumor, and 20% responded partially. In 28% of all patients that responded to Herceptin no reoccurrence of the tumor was observed within the first 12 months (166).

Although Herceptin treatment has been shown to be successfull there are some side effects. Cardiotoxicity has been reported to occur particularly when Herceptin is administered in combination with antineoplastic agents such as anthracyclines. ErbB2 has been demonstrated to be essential for prevention of dilated cardiomyopathy (167). Herceptin enhances tumor necrosis factor-mediated apoptosis in breast and ovarian cancer cell lines that overexpress ErbB2 (168), and is known to induce ADCC against ErbB2 overexpressing tumors, but ADCC against heart tissue has not been reported.

Another ErbB2-targeted monoclonal antibody, pertuzumab (Omnitarg, 2C4; Genentech), is currently being tested in Phase I clinical trials in cancer patients with different types of solid tumors. In contrast to trastuzumab, pertuzumab sterically blocks ErbB2 dimerization with other ErbB receptors and blocks ligand-activated signaling from ErbB1/ErbB2 and ErbB2/ErbB3 heterodimers (169). This characteristic might explain why pertuzumab inhibits the growth of tumors that express low levels of ErbB2, while trastuzumab does not (169).

Cetuximab is a chimeric antibody directed against ErbB1. It is FDA approved for treatment of colorectal cancer and is now in clinical trails for

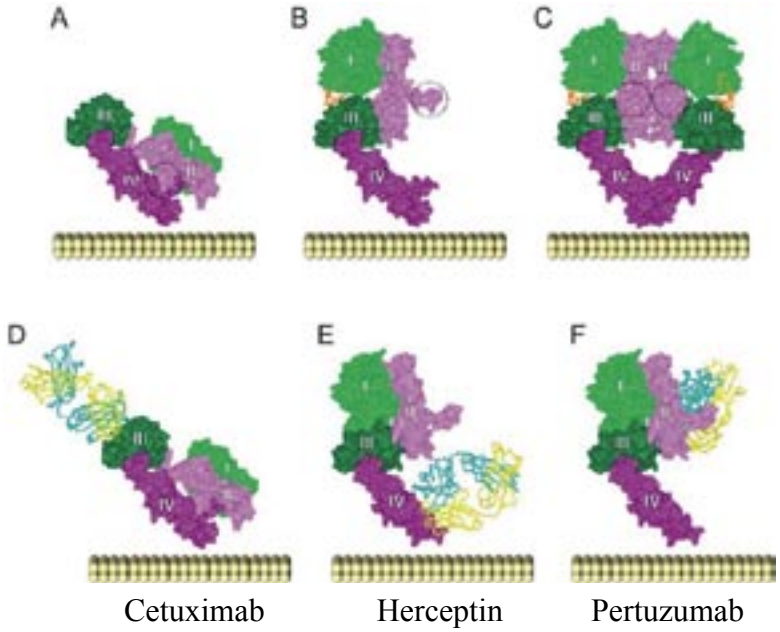

Figure 8. Ligand activation of ErbB1 and antibody targeting of ErbB1 and ErbB2. The ectodomains of ErbB1 and ErbB2 are shown in surface representation. The extracellular domains are numbered I to IV (L1, S1, L2 and S2 respectively). The dimerization arm in domain II is indicated by a dashed circle in **A, B and C**. The antibodies are indicated in **D, E and F**. The receptor ectodomains have been orientated such that the position of domain IV is common. The membrane bilayer is represented by the cylindrical structures. **A:** Structure of the unbound ErbB1 ectodomain (autoinhibited, tethered state). **B:** Structure of the 1:1 EGF:ErbB1 ectodomain complex (dimerization-competent state). **C:** Structure of the 2:2 EGF: ErbB1 ectodomain complex (dimerized, activated state). **D:** Structure of cetuximab bound to domain III (L2) of the ErbB1 ectodomain. **E:** Structure of Herceptin bound to domain IV (S2) of the ErbB2 ectodomain. **F:** Structure of pertuzumab bound to domain II (S1) of the ErbB2 ectodomain. Figure taken from Hubbard *et al.* (203).

treatment of patients with pancreatic cancer and HNSCC and NSCLC (170). Cetuximab interacts exclusively with domain III of ErbB1 on a site that partly overlaps with the ligand binding region. Furthermore it sterically prevents the receptor from adopting the extended conformation which is required for dimerization (171).

Tyrosine kinase inhibitors (TKIs) are small organic molecules, that compete with ATP for binding to the receptor kinase. Anilinoquinazoline derivates are both selective and effective inhibitors of the ErbB1 tyrosine kinase. Many of these inhibitors are being tested for the treatment of cancer including Tarceva and Iressa, which are currently approved for the treatment of NSCLC.

13 Tumor resistance to ErbB1 and ErbB2 directed drugs

Not all patients whose tumor overexpress ErbB1 or ErbB2 respond to Herceptin or Iressa. ErbB2 is highly phosphorylated in ErbB2 overexpressing breast cancer cells and the PI3K/Akt pathway is constitutively active even in absence of ligand. Because ErbB3 has a defective kinase domain, its role in tumorigenesis has long been underestimated. ErbB3 has multiple p85/p110α (subunit of PI3K) binding sites and forms the link between ErbB2 and the PI3K/Akt pathway, and may therefore play an important role in the resistance to Herceptin and Iressa. Knockdown of ErbB3 by siRNA in the human breast cancer SKBR3 cell line restored its sensitivity to the ErbB1-specific tyrosine kinase inhibitor Iressa (172). Herceptin causes dissociation of the ligand-independent constitutive ErbB2-ErbB3 heterodimer, thereby preventing the activation of the PI3K-Akt pathway (173). NRG1, however, prevents Herceptin from disrupting the ErbB2-ErbB3 heterodimer. Knockdown of NRG1 expression by siRNA blocked ErbB3 activation and subsequent PI3K-Akt activation, and sensitized MCF-7 cells to the topoisomerase II inhibitor doxorubicin (174, 175). Furthermore, Herceptin appeared unable to prevent Akt activation in cancer cells with activating mutations in PI3K or low expression levels of PTEN (176), suggesting that mutant PI3K and low PTEN are markers for poor patient response to Herceptin. Chemical inhibitors of PI3K, such as GDC-0941, act synergistically with Herceptin in inhibition of Akt activity (173). GDC-0941 also inhibits Akt activity in Herceptin-resistant tumor cells. PI3K inhibitors are in clinical trials and may hopefully prevent ErbB3-mediated resistance to Herceptin.

14 Role of the Drosophila epidermal growth factor receptor in development

The Drosophila epidermal growth factor receptor (DER) signaling complex consists of only a single receptor and four activating ligands: Spitz, Keren, Gurken and Vein. The EGF receptor system in flies is unique because in addition to these activating ligands also a secreted antagonist has been identified, named Argos. DER is expressed in almost every tissue in the fly and is a key regulator in its development by directing cell fate choices, cell division, cell survival and cell migration (reviewed (177)). In most cases DER is activated by ligands produced in adjacent cells, but DER can also be activated by a concentration gradient of DER ligand, in which the level of DER activation determines the cell fate choices (178-180). Unlike their mammalian homologues the DER activating ligands Spitz, Keren and Gurken are produced as large inactive transmembrane precursors (83, 181-183). The activation of these ligands is tightly regulated by the cleavage of the membrane-bound precursor and is known as a key step in DER activation. Ligand processing is controlled by the type II transmembrane protein Star and the seven-

transmembrane domain protease Rhomboid (184, 185). Star transports the ligand precursor from the endoplasmatic reticulum to the Golgi compartment (186) and Rhomboid is responsible for cleavage of the ligand precursor (184, 185). Secretion of DER ligands and subsequent DER activation is modulated by the compartmentalization of the three different Rhomboid members Rho1, Rho2 and Rho3 (187). Rho1 is broadly expressed and is concentrated in large patches near the apical membrane of the cell (188). Rho-2 and Rho-3 are expressed in the germ line and in the developing eye, respectively. In addition to cleavage of the ligand precursor, Rhomboid also cleaves Star (189). Cleavage of Star restricts the amount of DER ligand that is trafficked. Rho2 and Rho3 cleave the DER ligand precursor and Star already in the ER, making it impossible for Star to transport Spitz to the late compartments of the secretory pathway at the apical membrane. This property attenuates DER activation, primarily by reducing the amount of Star that can direct the ligand precursor to the late compartment, where cleavage of Spitz and subsequent secretion take place (187). Since the pattern of Rhomboid activation coincides with that of DER-induced MAPK-activation (190), regulation of Rhomboid expression appears the most important step in DER activation. The dynamic control of Rhomboid expression is very complex. The maternal morphogen Dorsal acts in concert with basic helix-loop-helix proteins, including Twist, to induce Rhomboid expression in both lateral and ventral regions. Expression is blocked in ventral regions (the presumptive mesoderm) by Snail, which is also a direct target of Dorsal (191). In addition activated DER induces Rhomboid expression (178), resulting in a positive feedback loop that gives rise to enhanced DER activation.

Vein is a large secreted ligand that does not need further processing. Spitz is involved in the development of almost all tissues. The role of Keren in development remains speculative. The only experimental evidence is that Spitz and Keren act redundantly in the development of the eye (192). Vein is involved in muscle development patterning of the ventral ectoderm and proximal-distal patterning in leg development. Vein is also a transcriptional target of DER, and since Vein is only a weak activating ligand it is most likely involved in the maintenance of a low activation level of DER at sites where no Spitz processing occurs. Activation of DER by Gurken is restricted to the ovary. Argos, is also a large secreted protein which is highly induced upon DER activation and provides a negative feedback loop in DER signaling (193). Argos is essential for the formation of several body axes, eye development, brain development and for the induction of bract cells in the developing leg. In addition to induction of Argos, three other negative feed back loops are involved in the attenuation of DER signaling, which are mediated by Kekkon, Sprouty and DCbl. Kekkon is a transmembrane protein that interferes with ligand binding and receptor dimerization (100). Sprouty inhibits the RAS/MAPK pathway, by direct interaction with Drk (Drosophila homologue of mammalian Grb2) and Gap1 (194), while DCbl induces ubiquitination of activated DER, resulting in endocytosis and degradation of the receptor (195). Unlike Argos,

Sprouty is also active in mammalian cells, while LRIG1 is the mammalian homologue of Kekkon1.

14.1 The antagonistic mechanism of Argos

For many years it has been thought that Argos acts as an antagonist of DER signaling by direct binding to the receptor and thereby preventing the binding of an activating ligand. Similarly to the agonistic ligands, Argos has an EGF-like domain which is essential and sufficient for its biological function, suggesting that Argos may directly interact with DER (196). Moreover, BIAcore measurements indeed showed that Argos binds with high affinity (17-41 nM) to DER (197). However, in 2004 Klein et al. (198) reported that Argos inhibits epidermal growth factor receptor signaling by sequestering the ligands, and recently the crystal structure of Argos in complex with Spitz has been published (199). This crystal structure demonstrated that Argos does not fold according to an EGF-like domain, which makes it unlikely that Argos competes with Spitz for interaction with DER. Instead Argos has a three-finger toxin fold (200) which clamps Spitz by covering 35% of the Spitz surface with a Kd of 7.7 nM (199). Based on the amino acid sequence no mammalian homologues of Argos have ever been identified.

THESIS OUTLINE

The ErbB signaling network is a complex system of four ErbB receptors, which are each activated by a different subset of in total eleven ligands. Depending on their ErbB binding specificity, EGF-like growth factors can be divided into three subfamilies: EGF, TGFA, AREG and EPGN form a subfamily that binds exclusively to ErbB1, while the subfamily consisting of BTC, EREG and HBEGF binds not only ErbB1, but also ErbB4. Finally the neuregulins form a subfamily of which NRG1 and NRG2 bind both ErbB3 and ErbB4, while NRG3 and NRG4 appear specific for ErbB4 (39). ErbB2 does not bind ligand by itself, but is the preferred heterodimerization partner for all liganded ErbBs and is particularly important for ErbB3 signaling which has a defective tyrosine kinase domain. Within each subfamily the various ligands can bind their receptor with different affinities: EGF, TGFA and AREG bind ErbB1 with high affinity while EPGN is a low affinity ErbB1 ligand. BTC and HBEGF are high affinity ErbB4 binders, while EREG binds ErbB4 with low affinity. Finally the β isoforms of NRG1 and NRG2 bind ErbB3 and ErbB4 homodimers with relative high affinities, while the α isoforms has low binding affinity for ErbB3 and ErbB4 which is stabilized upon heterodimerization with ErbB2 in order to interact with moderate affinities. The aim of the present study is to understand the molecular basis for ErbB specificity and affinity of EGF-like growth factors. In this context we posed the following questions:

1. What is the molecular basis of the ligand-receptor binding affinity?
2. What is the molecular basis of receptor specificity of ErbB ligands?
3. What mechanism determines hetero- versus homodimerization?
4. What is the relation between binding affinity and the mitogenic potential of a ligand?

Chapter 2 describes a phage display approach to investigate the sequential requirements for ErbB3 binding and subsequent ErbB2-ErbB3 heterodimer formation by ligands. In that study we used the EGF-TGFA chimera T1E as a template molecule because it binds with low affinity to ErbB3 homodimers and with high affinity to ErbB2-ErbB3 heterodimers. T1E variants with a modified C-terminus were selected in order to discriminate their ability to form ErbB3 homodimers from the formation of ErbB2-ErbB3 heterodimers. We observed that the ligands have no specific selectivity and the ErbB-3 affinity of a ligand determines whether it forms preferentially ErbB3 homodimers or ErbB-2/ErbB-3 heterodimers.

In **chapter 3** we postulate the concept that receptor affinity is controlled by ligand residues that are directly involved in receptor binding (positive constraints), while receptor selectivity is mainly controlled by evolutionary conserved residues that are not essential for high affinity binding, but prevent the undesired binding to other ErbB receptors (negative constraints). Using phage display we optimized positive constraints in the C-

terminal region of T1E for high affinity binding to ErbB1, ErbB3 and ErbB4, while negative constraints for ErbB3 and ErbB4 were released. Homology models of ErbB3 and ErbB4 identified R45 in EGF as a negative constraint preventing interaction with ErbB3 and ErbB4. Panerbin, a T1E selectant that binds with high affinity to all three receptors, proved to be an excellent tool for determining the relative amount of ErbB receptors on breast tumor cells.

Chapter 4 describes an EGF mutant with high binding affinity for ErbB3. This EGF mutant, designated WVR/EGF/IADIQ consist of a N-terminal and a C-terminal linear region which are optimized for ErbB3 binding. VWR/EGF/IADIQ still binds to ErbB1 and is able to activate ErbB2-ErbB3 heterodimers but not ErbB1-ErbB3 heterodimers while NRG can. VWR/EGF/IADIQ is the first reported ligand which has a higher affinity for ErbB3 than for ErbB4.

In **chapter 5** we demonstrate that positive constraints found in a T1E background are able to increase the binding affinity of natural ligands for their receptors, indicating that evolution has not selected ligands for their highest binding affinity. Furthermore we show that an increase of ErbB binding affinity does not necessary result in enhanced mitogenic activity. These observations are discussed in terms of sequential requirements for optimization of ErbB affinity, selectivity and mitogenic potential during evolution.

Chapter 6 describes the use of a radio-labeled high affinity NRG1β mutant, NRG/YYDLL, for determination of ErbB3 levels by receptor binding analysis. Furthermore, we show by differential competition with unlabeled NRG/YYDLL and BTC that the number of ErbB3 and ErbB4 receptors can be quantified separately on cultured human breast cancer cells.

Chapter 7 discusses why NRG1β does not interact with ErbB1. Based on homology models of NRG1β with ErbB3 or ErbB4, we identified the amino acids in NRG1β that are directly involved in ErbB3 or ErbB4 interaction. These models did not provide any indication for a putative steric clash or a charge repulsion that could prevent NRG1β from binding ErbB1. Using domain exchange studies we observed that the NRG1β A1-loop and C-loop sequences are involved in preventing ErbB1 binding.

Chapter 8 describes an attempt to humanize the DER agonist Spitz using a domain exchange approach with EGF. The binding characteristics of the EGF-Spitz chimeras tested on ErbB1 and DER provided information about the sequence requirements for high affinity binding to both receptors. However, the question why Spitz does not bind ErbB1 and why EGF does not bind DER remains unanswered.

In **chapter 9** the above data are discussed in terms of models for: 1) the acquirement of receptor specificity of ErbB ligands during the evolution; 2) the relationship between ErbB affinity and mitogenic activity; and 3) the role of negative constraints in receptor specificity of ErbB ligands.

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2

Selective formation of ErbB2/ ErbB3 heterodimers depends on the ErbB3 affinity of epidermal growth factor-like ligands

Journal of Biological Chemistry 278, 12055-12063 (2003)

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ABSTRACT

EGF-like growth factors activate their ErbB receptors by promoting receptor-mediated homodimerisation, or alternatively by the formation of heterodimers with the orphan ErbB2 through an as yet unknown mechanism. To investigate the selectivity in dimer formation by ligands, we have applied the phage display approach to obtain ligands with modified C-terminal residues that discriminate between ErbB2 and ErbB3 as dimerisation partner. We used the EGF/TGFα chimera T1E as template molecule, since it binds to ErbB3 homodimers with low, and ErbB2/ErbB3 heterodimers with high affinity. Many phage variants were selected with enhanced binding affinity for ErbB3 homodimers, indicating that C-terminal residues contribute to the interaction with ErbB3. These variants were also potent ligands for ErbB2/ErbB3 heterodimers, in spite of negative selection for such heterodimers. In contrast, phage variants positively selected for binding to ErbB2/ErbB3 heterodimers, but negatively for binding to ErbB3 homodimers, can be considered as "second best" ErbB3 binders, which require ErbB2 heterodimerisation for stable complex formation. Our findings imply that EGF-like ligands bind ErbB3 through a multi-domain interaction, involving at least both linear endings of the ligand. Apparently the ErbB3 affinity of a ligand determines whether it can form only ErbB2/ErbB3 complexes or also ErbB3 homodimers. As no separate binding domain for ErbB2 could be identified, our data support a model in which ErbB heterodimerisation occurs through a receptor-mediated mechanism, and not through bivalent ligands.

INTRODUCTION

The recent determination of the crystal structure of the extracellular domain of ErbB-1 in complex with its ligands epidermal growth factor (EGF) or transforming growth factor α (TGFα) has provided evidence for the formation of homodimeric ErbB1 complexes through a receptor-mediated dimerisation mechanism (1,2). Ligand binding to both domains I and III of the extracellular domain of the receptor involves the transition of ErbB1 from a 'closed' to an 'open' state, which then permits dimerisation with another liganded ErbB1 through interaction of domain II residues within these receptors. Most likely this mechanism can serve as a paradigm for homodimerisaton of other liganded ErbB receptors, such as ErbB3 and ErbB4. However, it is well established that EGF-like growth factors preferentially signal through heterodimers of their cognate receptor with the orphan ErbB2. The mechanism by which EGF-like growth factors bind their receptors in heterodimeric receptor complexes remains an open question.

Dimerisation of ligand-bound receptor tyrosine kinases is a mechanism that is thought to activate the intrinsic kinase domain, followed by transphosphorylation and subsequent docking of cellular signal

transducing proteins. As a consequence, ligand binding serves as a potential site for regulation of cell proliferation in diseases where ErbB receptors are overexpressed, as has been observed for ErbB-1 and ErbB2 in multiple human cancers (3). ErbB2 has no known ligand, but by decelerating the ligand dissociation rate it serves as a preferred dimerisation partner for all other ErbB members (4-6). Heterodimer formation with ErbB2 is especially important in the case of the ErbB3, which together with ErbB4 forms the natural receptor for the different neuregulins (NRGs). ErbB3 contains a defective kinase and hence ErbB3 homo-dimers are biologically inactive (7,8). The ErbB2/ErbB3 heterodimer, however, is the most prominent and strongest transforming signaling complex activated by NRG1 (9-12) and provides an attractive model system to study the mechanism of ligand-induced ErbB heterodimerisation.

EGF-like growth factors share a structurally conserved EGF motif, characterised by three disulphide-bonded loops (the A-, B- and C-loop), in addition to a linear N-terminal and C-terminal region. Structural and mutational analyses have shown that residues in the A-loop, C-loop and C-terminal linear region of EGF and TGFα primarily bind to domain III of ErbB1, followed by an interaction of residues in their B-loop with domain I of the receptor (1,2,13-16). By contrast, NRG-1β has been shown to bind with high affinity to a proteolytic fragment of ErbB3 containing only domain I (17). Moreover, NRG binding to ErbB1/ErbB4 chimeras requires the presence of domain I of the latter receptor, suggesting that ligand binding to NRG receptors primarily involves interaction with domain I of the receptor (18). Alanine scanning of NRG-1β revealed that hydrophobic and charged residues in the linear N-terminal region and the B-loop, that form a surface patch on one site of the triple β-sheet, are the major determinants in ErbB3 binding (19,20). On the other hand, also residues in the C-terminal region of NRG may play a role in receptor binding, since the natural α- and β- isoforms of NRG1 and NRG2, which only vary in sequences C-terminal of the fifth cysteine, strongly differ in their ability to bind and activate distinct ErbB combinations (21-24). Exchange studies between NRG1α and NRG1β have shown that particularly the linear C-terminal region determines the binding properties and mitogenic potential of these isoforms (25). Therefore it has been proposed that NRGs may have a bivalent character and interact with both ErbB3 and ErbB2 through separate binding sites (26).

To evaluate the contribution of residues in the linear C-terminal region of EGF-like ligands for selective dimer formation, we applied the phage display technique to select ligands that discriminate between ErbB-2 and ErbB-3 as dimerisation partner in ErbB3 complexes. In earlier work we and others showed that EGF chimeras in which the linear N-terminus was replaced by either NRG (biregulin) or TGFα (T1E) residues gained high binding affinity to ErbB2/ErbB3 heterodimers, while they bound only weakly to ErbB3 alone (27,28). Unlike NRG1β, both T1E and biregulin seem dependent on subsequent binding of ErbB2 to stabilise their low affinity interaction with ErbB3. In a previous study we could attribute the weak ErbB3 interaction

of the chimera T1E in part to the presence of sub-optimal sequences in the linear N-terminus. Based on a phage display approach we enhanced the binding affinity for ErbB-3 relative to T1E by substitution of only two residues in EGF (D2W and S3V/R)(29). In the present study we have used the same approach to subject five residues in the linear C-terminal region of T1E for randomisation and selection for altered receptor selectivity and affinity. The targeted residues in T1E correspond to the positions in the NRG isoforms that have been implicated in the differential activation of ErbB dimers. Here we show that T1E can be strongly optimised for binding to ErbB3 by the current phage display approach, indicating that residues in the linear C-terminal tail contribute to the ability of ligands to bind ErbB-3 homodimers. Moreover, in spite of negative selection protocols we consistently observed a direct relation between the ability of T1E variants to bind ErbB3 and their ability to induce ErbB2/ErbB3 heterodimers. Since no sequences selective for ErbB2 heterodimerisation could be identified, our findings support a model in which ErbB heterodimerisation is driven by receptor-mediated and not by ligand-mediated interactions.

RESULTS

The linear C-terminal region of T1E influences the ErbB2/ErbB3 binding efficiency

Previous work showed that the chimeric EGF-like ligands biregulin and T1E bind only weakly to ErbB3, due to sub-optimal sequences in the N-terminal linear region (26,29). The observed high affinity binding of T1E and biregulin for cells expressing both ErbB2 and ErbB3 thus appears to depend critically on additional stabilisation of the complex by ErbB2. The strong dependence on ErbB2 as dimerisation partner makes this type of molecules a good model system for identifying residues that mediate the selective recruitment of ErbB2 *versus* ErbB3 as dimerisation partner.

In this study we have examined whether sequences in the C-terminal linear region of T1E play a direct role in the formation of ErbB3 homodimeric and ErbB2/ErbB3 heterodimeric complexes, as suggested by a comparison of the different NRG isoforms. Thereto we initially exchanged the C-terminal linear tail of T1E, composed of EGF sequences, for the corresponding sequences of TGFα (Glu44-Ala50), NRG1β (Gln45-Ala51) and an optimised NRG1β mutant. This latter mutant has been obtained from a previous phage display study on NRG1β by selection for high affinity binding to ErbB3 ectodomains, and contains the mutations N47H and M50I compared to the wild-type NRG1β EGF domain (30). The TGFα C-terminus was included because it shows the highest sequence similarity with optimised NRG1β, and because previous studies have indicated that introduction of TGFα C-terminal residues into NRG-1α

strongly enhances binding affinity for ErbB2/ErbB3 heterodimers on SK-BR-3 cells (35). The nomenclature used for the T1E mutants follows previously used conventions, in which T1E6N is a chimera containing TGFα sequences up to the first cysteine, followed by EGF sequences up to the sixth cysteine and NRG-1β residues in the C-terminal tail (Figure 1A). All T1E mutants were expressed as recombinant peptides in *Escherichia coli*, finally purified by reverse-phase HPLC and verified for the appropriate molecular weight by MALDI-TOF analysis.

The biological activity of the various T1E mutants was assessed by competitive binding analysis on stable transfectants of 32D cells that express defined ErbB combinations (12). Figure 1B shows clear differences in the ability of the T1E mutants tested to displace radiolabelled T1E from 32D cells coexpressing ErbB2 and ErbB3 (D23 cells). The IC_{50} of both T1E6N and T1E6T (50-60 ng/ml) was increased compared to T1E itself (6.7 ng/ml), whereas T1E6N^{opt} bound D23 cells with enhanced affinity (3 ng/ml) comparable

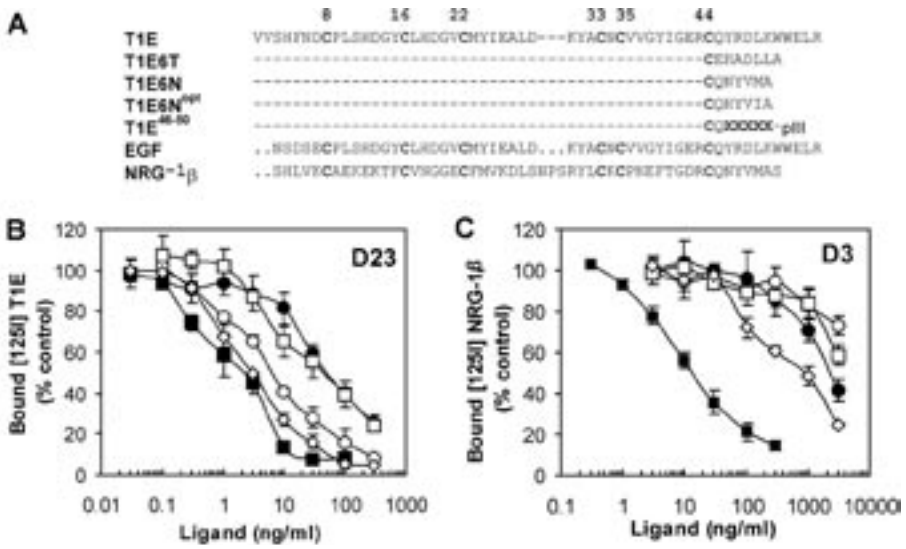

Figure 1: Exchange of the linear C-terminal region in the EGF/TGFα chimera T1E affects the binding to specific ErbB combinations. (**A**) Amino acid sequences of the T1E variants used in this study, aligned with human EGF and the EGF-domain of human NRG1β. Numbering of the conserved cysteines in *bold* face is according to T1E. (**B**) Displacement of [^{125}I] T1E binding on 32D cells coexpressing ErbB2 and ErbB3 (D23 cells). (**C**) Displacement of [^{125}I] NRG1β binding on 32D cells expressing ErbB3 (D3 cells). Cells were incubated for 2 hrs at 4°C with radiolabelled ligand (2 ng/ml) in presence of serial dilutions of unlabelled NRG1β (closed squares), T1E (open circles), T1E6T (closed circles), T1E6N (open squares), or T1E6N^{opt} (open diamonds). Unbound ligand was removed by sedimentation of the cells through a serum cushion, after which cell-bound radioactivity was determined. Results express the mean ± sem of three independent experiments performed in duplicate.

to wild type NRG-1β (2 ng/ml). Similar differences were observed in the ability of these T1E mutants to stimulate proliferation of D23 cells and the neuregulin-responsive MCF-7 human breast cancer cells, indicating that their relative binding affinity corresponded to the ability to activate ErbB-2/ErbB-3 complexes (data not shown). Thus, changes in the C-terminal sequences of T1E strongly affect its ability to interact with ErbB-2/ErbB-3 heterodimers. Despite the fact that NRG-1β is the natural activator for ErbB-2/ErbB-3 heterodimers *in vivo*, its C-terminal sequences do not appear beneficial for the interaction with ErbB-2/ErbB-3 in a T1E environment. In this respect the EGF residues present in T1E appear more effective in stabilising ErbB-2/ErbB-3 complexes than the corresponding NRG-1β and TGF-α sequences.

To further evaluate the role of the linear C-terminus in binding to ErbB-3, the binding affinity of the T1E mutants was assessed by [^{125}I] NRG-1β displacement on 32D cells solely expressing ErbB-3 receptors (D3 cells). In agreement with crosslinking analyses, we assume that NRG-1β is able to induce homodimeric ErbB-3 complexes in D3 cells (12,26). Figure 1C shows that all T1E mutants have an affinity for ErbB-3 alone that is at least 50-fold lower than that of NRG-1β. Compared to T1E, T1E6N^{opt} shows increased binding affinity, while T1E6N and T1E6T have almost similar binding affinity. Thus, replacement of the C-terminal tail of T1E for NRG-1β sequences did not significantly improve the weak binding of T1E to the ErbB-3 receptor. By contrast, introduction of the C-terminal tail of the optimised NRG-1β variant increased the relative binding affinity of T1E for both homo-and heterodimeric ErbB-3 complexes, indicating these residues improve the recruitment of ErbB-2 and ErbB-3 independent of the context of the NRG molecule. Together these findings demonstrate that residues in the C-terminal region of T1E indeed contribute to the ligand preferences for distinct receptor complexes.

Design of the T1E$^{46-50}$ phage library and selection strategy

To assess the precise contribution of individual residues in T1E to the recruitment of ErbB2 or ErbB3 into dimeric complexes, we randomly mutated five positions in the linear C-terminus of T1E that correspond to the positions in the NRG isoforms implicated in differential ErbB binding using a phage display approach. By a combination of positive and negative selection strategies and specific elution methods, we subsequently selected variants that discriminated between binding to ErbB3 homodimers and to ErbB2/ErbB3 heterodimers. To this end we used homodimeric ErbB3-IgG fusion proteins in addition to MDA-MB-453 cells, human breast carcinoma cells with overexpression of ErbB2 and ErbB3, which we have previously employed for affinity optimisation of EGF variants to ErbB2/ErbB3 heterodimers (29). Figure 1A shows the sequence of the phage library of T1E variants. The Gln45 residue adjacent to Cys44 is conserved between EGF and NRG1β, and therefore the adjacent five residues (Tyr46-Lys50) were targeted for

mutation. Because the randomised area is localised in close proximity to the fusion point with the pIII minor coat protein, a flexible linker was introduced into the fUSE5 phage vector to minimise possible steric effects. The resulting phages each display 2-5 copies of the fusion proteins (32). The completeness of the $T1E^{46-50}$ phage library was estimated from the number of independent transformants yielding 1.45×10^7, thereby covering five times the theoretical diversity of 3.1×10^6 possible different amino acid combinations. Sequence analysis of an aliquot of the library revealed no deviation from the theoretical amino acid distribution.

Sequences of $T1E^{46-50}$ clones isolated for preferential ErbB3 homodimer formation

Phage T1E variants that preferentially bind to ErbB3 homodimers but not ErbB2/ErbB3 heterodimers were isolated using alternating selection rounds on homodimeric ErbB3-IgG fusion proteins and whole MDA-MB-453 cells. During the selection on cells the ErbB2 dependent phage clones were depleted from the cell surface by competitive elution with anti-ErbB2 antibodies that are known to impair ligand binding (34). Subsequently the remaining phage clones bound to ErbB-3 receptors on the cell surface were harvested by acid elution. The alternation between exposure to soluble and cell-bound ErbB3 receptors reduced non-specific background binding of phages and excluded a possible selection advantage offered by the preformed dimeric ErbB3-IgG compared to the monomeric cell-expressed receptors.

Individual phage clones were randomly picked after four selection rounds and subsequently analysed for binding to ErbB3 ectodomains in phage ELISA. Table 1 gives a survey of the sequences of 16 $T1E^{46-50}$ clones selected for preferential binding to ErbB3 homodimers (ErbB3 selectants) with their corresponding binding properties. Binding ability of the clones to ErbB2/ErbB3 heterodimers was determined in ELISAs on intact cells (D23 *versus* parental 32D cells) and on heterodimeric ErbB2/3-IgG fusions. Since these heterodimeric IgG fusion proteins were generated by cotransfection of the expression vectors encoding the respective receptors, experiments were carried out on a mixture of ErbB2/3-IgG heterodimers and ErbB2-IgG and ErbB3-IgG homodimers. As positive control EGF/W2V3 phage was used, a previously characterised EGF variant with high affinity for ErbB3 homodimers and ErbB2/ErbB3 heterodimers (29). Wild-type T1E phage and EGF phage were used as additional controls. Table 1 indicates that all ErbB3 selectants displayed strong binding to ErbB3-IgG, with concomitant strong binding capacity to both ErbB2/3-IgG ectodomains and D23 cells. Thus, despite the negative selection for ErbB2 dimers, the T1E clones selected by this approach had not lost their ability to bind to ErbB2/ErbB3 heterodimers. In other words, the ErbB3 selectants are unable to discriminate between ErbB2 and ErbB3 as dimerisation partner.

TABLE 1
Sequences and binding characteristics of ErbB-3 selectants

Sequence T1E[46-50]	Clone binding[a]							
	46	47	48	49	50	n = 16	D23	ErbB-23-IgG	ErbB-3-IgG
3.1	Ile	Phe	Asp	Trp	Ala	5	++	++	++
3.2	Ile	Phe	Asp	Phe	Leu	1	++	++	++
3.5	Ile	Tyr	Asp	Val	Asp	1	++	++	++
3.4	Thr	Tyr	Asp	Phe	Pro	1	++	++	++
3.5	Tyr	Tyr	Asp	Ile	Asp	1	++	++	++
3.6	Tyr	Ile	Gln	Leu	Ser	2	++	++	++
3.7	Ile	Ala	Asp	Val	Gly	2	++	++	++
3.8	Ile	Ala	Asp	Ile	Gln	1	++	++	++
3.9	Ile	Ala	Asn	Met	Met	1	++	++	++
3.10	Ile	Trp	Asp	Phe	Pro	1	++	++	++
Consensus	Ile	Ar[b]	Asp	Ar[c]	X				

[a] Single phage clones were screened for binding in phage ELISA on D23 cells versus parental 32D cells and on ErbB-IgG fusion proteins. Relative binding was measured in comparison to the positive control phage EGFWT. ++, Signal intensity >100% relative to control.
[b] Preferential selection of aromatic (W/F/Y) or hydrophobic residues. Consensus sequence is based on a frequency occurrence of ≥50%.
[c] Preferential selection of residues with an aromatic (F/Y) or hydrophobic character.

The most striking result in the sequences obtained was the abundant selection of Asp48 in 81% of the ErbB3 selectants, which is the corresponding residue in both EGF and TGFα. Substitutes found on positions 46, 47 and 49 tended to be similar in character, being predominantly large and hydrophobic, although at position 46 a preference for Ile was observed (75%). At position 47 residues Tyr and Phe were found in more than half of the ErbB3 selectants, indicating that aromatic side-chains seem to be favoured. The most frequently selected T1E$^{46\text{-}50}$ clone, designated 3.1, contained IFDWA sequences (5/16 times). The overall consensus sequence for T1E$^{46\text{-}50}$ ErbB3 selectants, based on the frequency of occurrence of a certain (type of) amino acid detected at the respective position in ≥ 50% of all individual clones analysed, was I46-aromatic47-D48-apolar49-X50 in which X represents any residue.

Sequences of T1E$^{46\text{-}50}$ clones isolated for selective ErbB2/ErbB3 heterodimer formation

In an inverse strategy, phage T1E variants were isolated that selectively bound to ErbB2/ErbB3 heterodimers but failed to bind to ErbB3 homodimers. Thereto negative selection on homodimeric ErbB3-IgG fusion proteins was performed prior to the selection on MDA-MB-453 cells. Ligands that ultimately depended on ErbB2 for high affinity binding were eluted from the cell surface by treatment with ErbB2 antibodies. Control cell pannings confirmed the specific elution of wild-type T1E phages by this method (data not shown). After four rounds of selection, single clones were sequenced and analysed for binding properties in ELISAs in a similar way as described above for the ErbB3 selectants (Table 2). All T1E clones selected for preferential ErbB2/ErbB3 binding (ErbB-23 selectants) were found to bind strongly to both D23 cells and ErbB23-IgG ectodomains, confirming that selection had been achieved. When the ErbB2/3 selectants were analysed for their ability to bind to ErbB3 homodimers, half of the clones lacked detectable binding for ErbB3-IgG, similar to phage T1E (group 1), while the remaining were still

TABLE II
Sequences and binding characteristics of ErbB-2/ErbB-3 selectants

Sequence T1E[46-50]	Binding[a]							
	46	47	48	49	50	D23	ErbB-23-IgG	ErbB-3-IgG
1								
23.1	Phe	Leu	Thr	Val	Asp	+	++	+/−
23.2	Tyr	Leu	Thr	Leu	Asp	++	++	+/−
23.3	Tyr	Leu	Ser	Thr	Asp	+	++	−
23.4	Tyr	Leu	Ala	Leu	His	+	++	−
23.5	Tyr	Leu	Gln	Met	Asn	+	+	−
23.6	Tyr	Tyr	Leu	Ile	Asp	++	+	+/−
23.7	Tyr	Tyr	Gly	Phe	Asp	++	+	+/−
23.8	Trp	Tyr	His	Val	Asp	+	+/−	−
23.9	Ala	Tyr	Asp	Ile	Tyr	+	+	−
23.10	His	Leu	Asp	Leu	Leu	+	+/−	−
23.11	Tyr	Leu	Asp	Pro	Asn	+	+/−	−
23.12	Thr	Val	Gln	Gly	Gln	+/−	+	−
Consensus	Tyr	Leu	*X*	Asp[b]	Asp			
2								
23.13	Ile	Phe	Asp	Trp	Ala	++	++	++
23.14	Ile	Phe	Asp	Trp	Leu	++	++	++
23.15	Tyr	Tyr	Asp	Ile	Asp	++	++	++
23.16	Tyr	Leu	Glu	Ile	Asp	++	++	++
23.17	Tyr	Leu	Gln	Leu	Glu	++	++	+
23.18	Tyr	Leu	Asp	Ile	Ser	++	++	+
23.19	Tyr	Leu	Ser	Met	Trp	++	++	+
23.20	Ala	Tyr	Asp	Ile	Pro	++	++	+
23.21	Val	Ala	Asp	Ile	Pro	++	+	+
23.22	Glu	Tyr	Asp	Pro	Tyr	+	++	++
23.23	Trp	Leu	Asp	Pro	Leu	++	++	++
23.24	Trp	Asn	Asp	Pro	Gln	+	++	+
23.25	Trp	Val	Ser	Leu	Asn	+	+	+
Consensus	Ar[c]	Ap[d]	Asp	Ap[e]	*X*			

[a] Single phage clones were screened for binding in phage ELISA on D23 cells versus parental 32D cells and on ErbB-IgG fusions. Relative binding was measured in comparison to the positive control phage EGF/WV. ++, Signal intensity >100%; +, signal >80%; +/−, signal ≥50%; −, signal <50% of control.
[b] Preferential selection of residues with a hydrophobic character (9/12). Consensus sequence is based on a frequency occurrence of ≥50%.
[c] Preferential selection of aromatic (9/13) or hydrophobic residues.
[d] Preferential selection of hydrophobic residues (12/13).
[e] Preferential selection of hydrophobic residues (of which 7/13 are Leu/Ile).

able to bind with gradual affinity to ErbB3 homodimers (group 2). Thus, only a fraction of the ErbB2/3 selectants was able to discriminate against ErbB3 as dimerisation partner in the ErbB3 complex.

When comparing the sequences of the two distinct groups of ErbB2/3 selectants, both differences and similarities became apparent (Table 2). The consensus sequence of group 1 ErbB2/3 selectants could be assigned as Y46-L47-X48-apolar49-D50, while the consensus sequence of group 2 strongly resembled that of the ErbB3 selectants in Table I. The diminished ability to bind to ErbB3 homodimers of the group 1 clones correlated with a shift of the acidic Asp from position 48 (72% group 2) to position 50 (52% group 1). Predominantly large hydrophobic residues were found at positions 46, 47 and 49 in all ErbB2/3 selectants, similarly as observed for the ErbB3 selectants, although the exact nature of the side-chains differed. Taken together, the overall consensus sequence of ErbB2/3 selectants displays remarkable overlap with the consensus sequence for ErbB3 selectants.

Selected T1E$^{46-50}$ variants display gradual differences in ErbB3 binding

To gain further insight into the relative contribution of specific amino acids to the ability of ligands to bind ErbB3, we subjected a number of selected T1E$^{46-50}$ phage variants to extended analysis using dose-response

experiments. Based on the similarity and divergence from the consensus sequences, individual T1E selectants that harbour acidic Asp residues (at position 48, 50, or at both positions) combined with distinct hydrophobic residues were chosen and produced as large-scale cultures. Phage T1E served as a positive control for ErbB2/ErbB3 binding, phage EGF/WV as a

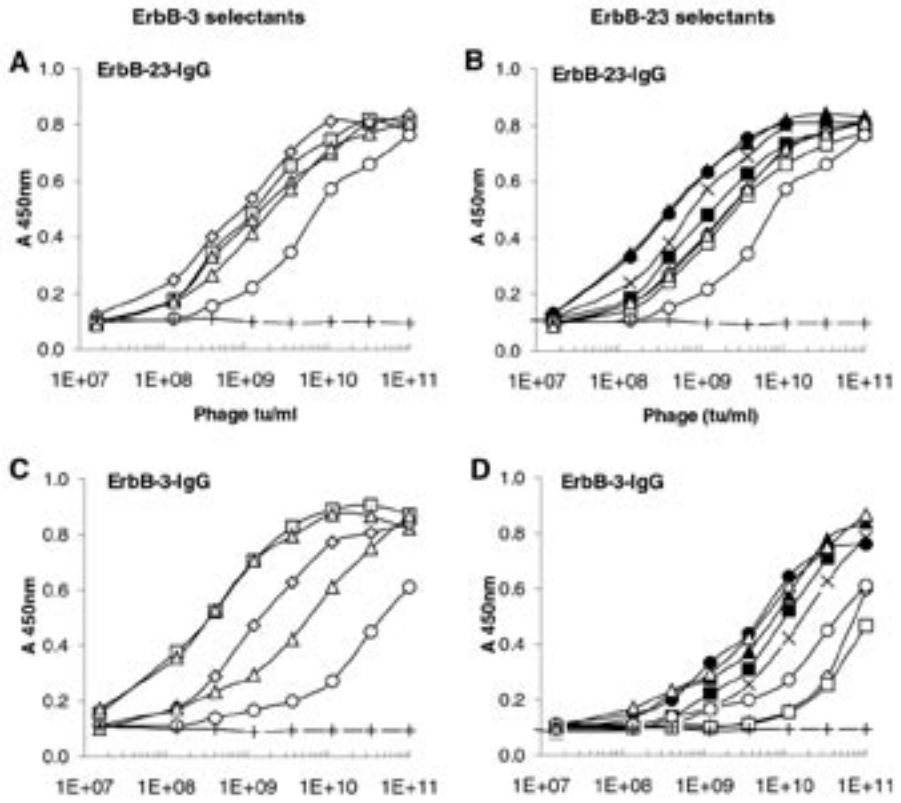

Figure 2: Binding characteristics of individual phage T1E$^{46-50}$ variants selected for preferential ErbB3 homodimer binding (ErbB3 selectants) or preferential ErbB2/ErbB3 heterodimer binding (ErbB2/3 selectants). (**A, B**) Phage ELISA on ErbB2/3-IgG fusion proteins. (**C, D**) Phage ELISA on homodimeric ErbB3-IgG fusion proteins. Binding of ErbB3 selectants is depicted in panel (A, C), ErbB2/3 selectants in panel (B, D). Phage clones used were T1E$^{46-50}$ variants IFDWA (grey squares), IADIQ (grey triangles), YYDID (grey diamonds), YLEID (closed circles), YLDIS (closed squares), YLQMN (closed triangles), YLTLD (crosses), YLALH (open diamonds), and YLSTD (open squares), while phage T1E (open circles), phage EGF/W2V3 (open triangles), and phage EGF (plusses) were used as positive and negative controls, respectively. Results are presented as mean of two experiments performed in duplicate.

positive control for ErbB3 homodimer binding, while phage EGF was used as a negative control.

While no absolute binding affinities could be determined by the used multivalent phage display system, the relative affinities could readily be compared between the distinct clones. All clones, invariably whether they were isolated as ErbB3 selectants (Figure 2A) or as ErbB2/3 selectants (Figure 2B), were found to bind with similar strong affinity to heterodimeric ErbB2/3-IgG complexes. The binding of wild-type T1E to the ErbB2/3-IgGs was relatively low in comparison to all other T1E variants and EGF/WV. This may be attributed to the flexible linker region which is present in the $T1E^{46-50}$ phages but absent in the wild-type T1E phage. Conversely, the binding affinity of the various $T1E^{46-50}$ clones to ErbB3-IgGs showed strong variation between the clones, and in general the ErbB3 selectants (Figure 2C) were superior to the ErbB2/3 selectants (Figure 2D). Interestingly, the ErbB3 selectants with C-terminal sequences IFDWA, IADIQ and YYDID showed significantly higher affinity for ErbB3 than the positive control EGF/WV (Figure 2C), indicating that C-terminal sequences strongly contribute to enhanced ErbB3 affinity. When comparing the ErbB-23 selectants, a more or less gradual decrease in ErbB3 binding affinity was observed in the order: YYDID > YLEID = YLQMN = EGF/WV > YLDIS > YLTLD > T1E > YLSTD and YLALH >> EGF (Figure 2D). Thus, acidic residues seem to contribute directly to the ErbB3 binding affinity when located at position 48, and to a lesser extent when present at position 50. Moreover, acidic residues present at both positions seem beneficial for ErbB-3 binding, while also the combination of Q48/N50 was shown to be efficient. Apparently the depletion of the strongest ErbB3 binding clones from the pool of ErbB2/ErbB3 selectants has resulted in the isolation of the "second best" ErbB-3 binding clones.

In addition, the abilities of the distinct T1E variants to activate ErbB2/ErbB3 heterodimers were assessed by measuring the IL-3 independent proliferation of D23 cells in an MTT assay. Figure 3 shows that all T1E variants are equipotent to phage EGF/WV and superior to phage T1E itself in inducing proliferative responses in D23 cells, indicating that functional ErbB2/ErbB3 heterodimers are formed in response to all selectants. Thus, the ErbB23 selectants only ineffectively bind to ErbB3 homodimers, similar to T1E, but are still strong inducers of ErbB2/ErbB3 heterodimers. The ErbB3 selectants effectively bind to ErbB3 homodimers and ErbB2/ErbB3 heterodimers, similar to EGF/WV, but this increased ErbB3 affinity does not result in enhanced mitogenic potency for cells expressing ErbB2 and ErbB3. Therefore it appears that a low threshold ErbB3 binding is already sufficient for the efficient formation of ErbB2/ErbB3 heterodimers.

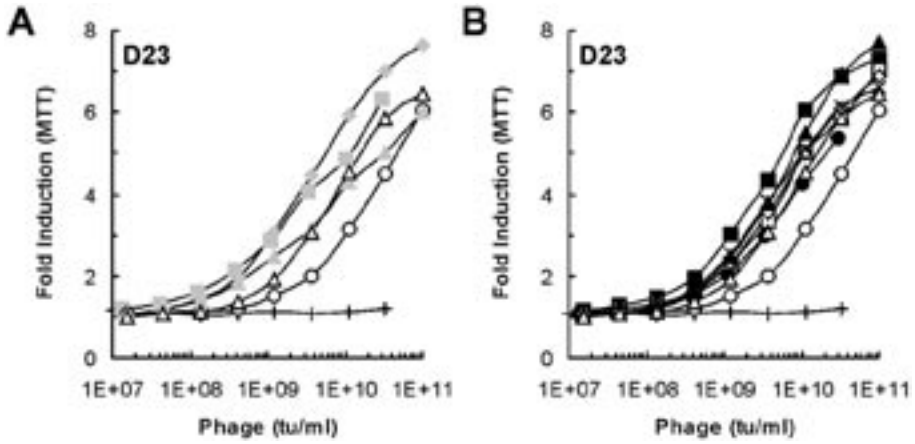

Figure 3: Ability of individual phage clones to induce proliferation of 32D cells expressing ErbB2 and ErbB3 (D23 cells). Cells were incubated for 24 hours in IL-3 free medium containing serial dilutions of filter-sterilised T1E$^{46-50}$ phages selected for ErbB3 homodimer binding (**A**) or ErbB2/ErbB3 heterodimer binding (**B**)**:** IFDWA (grey squares), IADIQ (grey triangles), YYDID (grey diamonds), YLEID (closed circles), YLDIS (closed squares), YLQMN (closed triangles), YLTLD (crosses), YLALH (open diamonds), YLSTD (open squares), or phage T1E (open circles), phage EGF/W2V3 (open triangles), and phage EGF (plusses). Viable cells were determined using the calorimetric MTT assay. Results are given as fold induction over non-stimulated cells of duplicate measurements.

DISCUSSION

EGF-like ligands differ in their ability to recruit a dimerisation partner for their cognate receptor, resulting in differential potency and mitogenic responses. This suggests that ligands contain specific residues that mediate interaction with distinct ErbB complexes, including heterodimers with the orphan ErbB2. To investigate the selectivity in dimer formation by EGF-related ligands, we have applied the phage display approach to obtain ligands with modified C-terminal residues that have (i) altered selectivity and (ii) enhanced binding affinity. Our findings indicate that EGF-like growth factors contain multiple, independent binding domains for ErbB3, one of which is located in the C-terminal tail. However, no separate binding domain for ErbB2 could be identified in this region. Instead, ligand-induced ErbB2/ErbB3 heterodimerisation appears to occur as a consequence of a low affinity interaction with ErbB3 and subsequent stabilisation by ErbB2.

To address the issue of dimer selectivity, distinct combinations of positive and negative selection strategies were applied to isolate ligands that were able to discriminate between ErbB2 and ErbB3 in complex formation with

ErbB3. Two of our present observations argue against the hypothesis that the selectivity in recruitment of the dimerisation partner is mediated by sequences in the linear C-terminal tail of ligands. First, none of the ErbB3 selectants had impaired ability to form ErbB2/ErbB3 heterodimers, in spite of the negative selection for T1E variants that depended on ErbB2 dimerisation for binding. The most likely explanation is that the linear C-terminal region is not directly involved in the recruitment of ErbB2 as dimerisation partner, either because ligands do not harbour a separate ErbB2 binding site, or because such a site is located in a different region of the ligand. Hence, our data do not favour a model in which the linear C-terminal tail harbours a secondary binding site for ErbB2, as previously proposed in ligand bivalence models (26,36).

Secondly, the incomplete discrimination against ErbB3 homodimer formation observed for the ErbB2/3 selectants might indicate that ligands require only a low level of ErbB3 binding affinity to allow the efficient formation of ErbB2/ErbB3 heterodimers. These ligands can thus be considered as the 'second best' ErbB3 binders. Moreover, the observation that ligand variants positively selected for ErbB3 binding, but negatively for ErbB2/ErbB3 binding, are still potent activators of heterodimers also strongly indicates that binding to ErbB3 is sufficient for heterodimer formation. It thus appears that the relative binding affinity to ErbB3 is indicative for the binding behaviour of ligands to dimeric complexes, such that low affinity binders only interact with ErbB2/ErbB3 heterodimers, while high affinity binders can additionally form ErbB3 homodimers.

Our present observations argue for a separate ErbB3 binding site located in the linear C-terminal tail of EGF-like ligands. Previous mutagenesis studies have assigned several non-continuous hydrophobic and charged residues in the triple β-sheet formed by the linear N-terminal region and the B-loop region of NRG1 as the major determinants for ErbB3 binding (19,20,27). It can however not be excluded that additional residues in the ligand may contribute to receptor interaction. For instance, residues in the β-turn in the A-loop of NRG1 were also susceptible to Ala mutation, although this might be attributed to structural disturbance as well (20). The crucial importance of N-terminal residues was further emphasised by two phage display studies, revealing the strong requirement for aromaticity (His or Trp) combined with aliphatic or basic side chains for ErbB3 interaction (29,30). Here we show that optimised sequences in the C-terminal linear region also directly contribute to the ability of T1E ligands to interact with ErbB3, and that they can compensate for the presence of suboptimal residues in the linear N-terminus of T1E. Notably, the $T1E^{46\text{-}50}$ variants selected for prefential binding to ErbB3 homodimers displayed even stronger binding to ErbB3-IgG than the EGF/W2V3 mutant (Figure 2C). Thus, while residues in the N-terminus and B-loop appear to confer specificity to ErbB3 binding, residues in the linear C-terminal tail may further enhance receptor affinity, in line with the differential ErbB-3 binding abilities of the distinct NRG1 isoforms. This suggests that both linear regions of the ligand participate independently in receptor binding,

such that ligand binding to ErbB3 occurs through a multi-domain receptor interaction.

The C-terminal sequence requirements for ErbB3 binding can be deduced from a comparison of the consensus sequences of the selected high and low affinity ErbB3 binding variants. Large hydrophobic residues are preferred at positions 46, 47 and 49 in all selected $T1E^{46-50}$ variants, while methionine was alternatively allowed at position 49. In particular Ile46 contributes to enhanced ErbB-3 binding affinity. In addition, the presence of an acidic residue facilitates specific binding to ErbB3, preferentially located at position 48 (for strong ErbB3 binding), or alternatively at position 50 (for weak ErbB3 binding). Since a combination of the polar residues Gln48 and Asn50 proved also sufficient (Figure 2D), we propose that these two side-chains are involved in hydrogen bonding. Interestingly, in the structure of receptor-bound TGFα several C-terminal residues interact with both domains I and III, among which the corresponding Asp47 in TGFα (1). It has been suggested that these residues determine the final position of the two ligand-binding domains in the complex, and thereby influence the stability of the dimer. It is tempting to speculate that in the T1E selectants Asp48 may serve a similar function when binding to ErbB3. Furthermore, all T1E variants selected for ErbB3 binding had maintained their ability to bind to ErbB-1 expressed on 32D cells and ErbB-1-IgG ectodomains, indicating that the requirements in the C-terminal tail for ErbB1 and ErbB3 interaction may partly overlap (unpublished observations). This observation is remarkable since the majority of selected clones lacked a Leu or Ile at position 49 in T1E (Leu47 in EGF), which is strictly conserved among ErbB1 ligands and known to be highly sensitive to site-directed mutagenesis (13,14).

Comparing the consensus sequence of the ErbB3 selectants with that of the natural ErbB3 ligands, one can conclude that the EGF linear C-terminal region comprising Tyr46-Asp48-Leu49 contributes positively to the interaction with ErbB3. The finding that T1E effectively forms ErbB2/ErbB3 heterodimers but not ErbB3 homodimers can thus not be attributed to inappropriate C-terminal sequences, as previously thought. Rather, it reflects the presence of suboptimal residues in both its N-terminal and C-terminal region (29). The observation that introduction of the NRG1β C-terminus into T1E did not increase the ErbB3 binding affinity can be explained by the presence of only two out of four required determinants, Tyr47 and Met49, with Met being a less-favoured residue. In agreement, mutation of these two residues into alanine was found to reduce the ErbB-3 binding affinity of NRG1β (20). Apparently, natural ligands are not necessarily optimised for high affinity to their receptors, which is also indicated by the increase in ErbB3 binding affinity of optimised NRG1β variants (30). Their affinity enhancement was mostly attributed to the Met to Ile substitution in the C-terminal tail, as also shown by the $T1E6N^{opt}$ mutant in our study. Interestingly, despite the abundant occurrence of Asp residues in the T1E variants optimised for ErbB3 binding, acidic residues were not observed in optimised NRG1β variants,

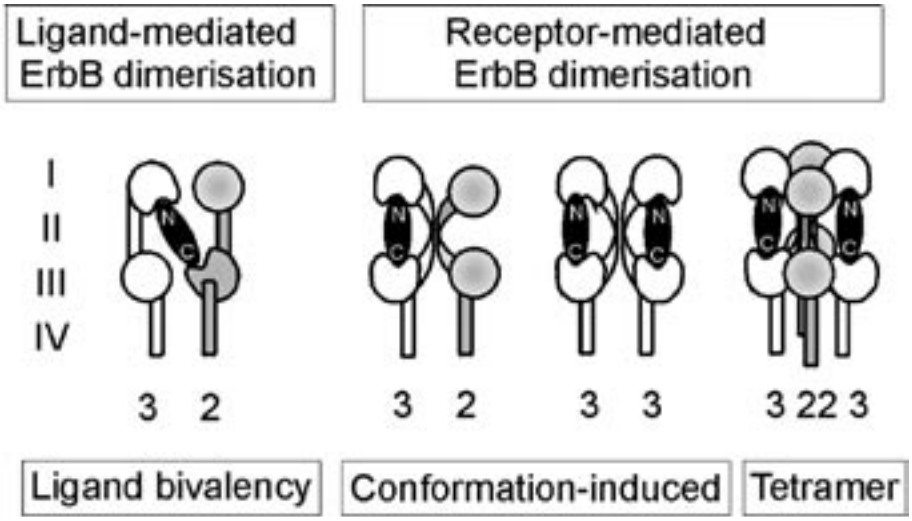

Figure 4: Models for ligand-induced ErbB2/ErbB3 heterodimerisation, including ligand-mediated (left panel) and receptor-mediated (middle and right panel) mechanisms. The ErbB3 receptor is shown in white and ErbB2 in grey. The orientation of the linear N-and C-terminal regions of the ligand is indicated by N and C, respectively. The middle panel depicts the ErbB2/ErbB3 heterodimeric complex induced by a ligand with low ErbB3 affinity, and the ErbB3 homodimeric complex induced by a ligand with high ErbB3 affinity. For details see text.

which may be attributed to differences in the structural environment of T1E and NRG1β (30). We have recently solved the three-dimensional structure of T1E, but since that of the NRG1β isoform is unknown, a direct comparison is impossible.

Our results imply that specific residues in the C-terminal tail of EGF-related ligands are involved in direct interaction with ErbB3 but not ErbB2. This makes it unlikely that the formation of heterodimeric ErbB complexes is driven through bivalent binding of ligands to two different ErbB molecules. Instead, our findings support a receptor-mediated dimerisation mechanism, in which ligand binding to ErbB3 determines the formation of both ErbB3 homodimers and ErbB2/ErbB3 heterodimers (Figure 4). In a variant of this model, it has been proposed that ligands only induce ErbB3 homodimers, which may subsequently stabilise two ErbB2 molecules into a tetrameric complex (37-40). Based on the analogy of the crystal structures of ErbB1 and ErbB3, it is most likely that homodimeric ErbB3 complexes are formed through a conformation-induced mechanism with a 2:2 stoichiometry (1,2,41). In this scenario the ligand would bind domain I of ErbB3 through the linear N-terminal and B-loop regions, while its C-terminal residues would interact with receptor domain III. In the conformation-induced model, selectivity between the formation

of inactive ErbB3 homodimers and active ErbB2/ErbB3 heterodimers will be achieved through the intrinsic property of a ligand to bind ErbB3 (Figure 4). Ligands with high affinity for domain I and/or III of ErbB3, including NRG1β and some of the ErbB3 variants selected here, have a high potency to bring ErbB3 into the "open" dimerisation state and will thus induce both ErbB3 homodimers and ErbB2/ErbB3 heterodimers. Ligands with relatively low affinity for ErbB3 due to suboptimal interaction with domain I or III, such as T1E and NRG1α, have only a low potency to induce ErbB3 into the "open" state and only in the case the liganded ErbB3 is complexed by ErbB2 a stable complex is formed. This model presumes that the dimerisation site of ErbB2 is maintained constitutively in the "open" configuration, as an explanation for the preferential heterodimerisation with ErbB2. From an evolutionary point of view this would be a sensible mechanism to enhance the formation of active ErbB2/ErbB3 complexes and to avoid biologically inactive ErbB3 homodimers. In this respect NRG1β would be the optimal natural ligand if ErbB2 is present in excess over ErbB3, while the low affinity NRG1α isoform would be more effective under conditions that ErbB3 is present in excess over ErbB2 (21,24). A switch in isoform production thus offers the cell a subtle method of control over cellular functions.

EXPERIMENTAL PROCEDURES

Construction of T1E mutants

The construction of T1E has been described previously (28). Residues in the linear C-terminal region following the sixth cysteine in pEZZ/Fx/T1E were replaced by the corresponding residues of TGFα and NRG1β by means of splice overlap extension polymerase chain reaction. The initial PCR fragments containing the complementary overhang sequences were made using pEZZ/Fx/T1E, pEZZ/Fx/TGFα and the NRG1β gene in pNRG8-g3 (a gift from Genentech Inc., San Francisco, CA) as templates. The fragment for the C-terminal region of the optimised NRG1β (referred to as NRG-58 (30)) was constructed using oligonucleotide primers containing mutations encoding Asn-His and Met-Ile substitutions. Mutant gene products were subsequently introduced in the pEZZ vector using the Bam-HI/Sal-I sites and verified by automated cycle sequencing. Production, purification and characterisation of recombinant T1E mutants was essentially performed as previously described (28).

Cell lines

Interleukin-3 dependent murine (m) 32D haematopoietic progenitor cells transfected with distinct human ErbB-encoding viral vectors or plasmids were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS, GibcoBRL Life Technologies, Paisley, UK), 0.25 ng/ml mIL-3 (Promega, Madison, WI), and kept under continuous selection using 0.6 mg/ml G418 (Calbiochem, La Jolla, CA) or 0.4 mg/ml hygromycin B (Sigma, St Louis, MO)(12). The human mammary carcinoma cell line MDA-MB-453 was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% FCS. Human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% FCS.

Ligand Displacement Experiments

Recombinant human NRG1$\beta^{176-246}$ (R&D Systems, Minneapolis, MN) and T1E were radioiodinated using the Iodogen method (Pierce) according to the manufacturer's protocol for indirect labelling, resulting in a specific activity of 40-80 µCi/µg protein. Ligand displacement analyses on 32D cells were performed as described (28).

Construction of the phage T1E$^{46-50}$ library

A phage library of T1E randomised by mutation to NNS codons (N=G/T/A/C, S=G/C) at positions Tyr46, Arg47, Asp48, Leu49 to Lys50 (T1E numbering) was constructed by a PCR-based approach using fUSE5/T1E as template (29,31). The randomised region in the peptide growth factor was directly followed by the *Sfi*-I restriction site, thereby omitting the EGF residues Trp51 to Arg55. Since the randomised sequence was located in close proximity of the pIII fusion point, a flexible $(Gly\text{-}Gly\text{-}Gly\text{-}Ser)_2$ linker sequence was introduced in the wild-type fUSE5 vector after the second Sfi-I site prior to the gene encoding pIII. Wild-type fUSE5 contains an out-frame stuffer fragment between the Sfi-I sites, thereby eliminating background phage (32). The PCR fragments encoding the randomised T1E gene were cloned into the fUSE5/linker phage vector using both *Sfi*-I sites. Ligation products were processed and electroporated into *Escherichia coli* TG-1 cells (Stratagene) for phage production. The number of independent transformants was determined by titration on tetracycline-containing plates to estimate the size of the library. Randomly picked clones from the library were analysed by cycle sequencing (Perkin-Elmer, Applied Biosystems) to confirm the diversity of

codon use and the expected amino acid distribution. Phage preparations were carried out following standard polyethylene glycol 8000/NaCl precipitation procedures. Titers of filter-sterilised phages were estimated by both titration and spectrophotometrical determination.

Preparation of ErbB-IgG fusion proteins

Gene constructs encoding the extracellular domain of human ErbB receptors were fused to the hinge and Fc regions of the human IgG1 heavy chain (referred to as ErbB-IgG (33)). Subconfluent HEK-293 cells were transfected with the expression vector pCDM7/IgB3 or a mixture of pCDM7/IgB3 with pCDM7/IgB2 using Lipofectamine2000 (GibcoBRL) according to the manufacturer's protocol. Conditioned culture supernatants containing the soluble dimeric IgG fusion proteins were harvested 5-10 days following transfection, and purified by affinity chromatography on a 1 ml Hi-trap Protein A-Sepharose column (Pharmacia Amersham Biotech). Purified IgG fusion proteins were eluted with 0.1 M citric acid (pH 4.2) into tubes containing 1 M Tris pH 9.0. ErbB-IgG preparations were quantified by Fc-ELISA using human IgG as a standard, while the purity and presence of dimeric species was confirmed by SDS-polyacrylamide electrophoresis and immunoblotting with polyclonal antibodies directed against human Fc (Nordic, Tilburg, NL).

Phage selection on ErbB3-IgG fusion proteins

Nunc immunoabsorbant wells were precoated overnight at 4°C with 0.2 µg of goat-anti-human Fc-specific IgG (Jackson Immunoresearch Laboratories, West Grove, PA) in 100 µl PBS (137 mM NaCl/ 2.7 mM KCl/ 1 mM Na_2HPO_4/ 2 mM KH_2PO_4). Wells were washed in PBS/0.05% (v/v) Tween20 (washing buffer) and blocked for 1 h in 0.2 ml PBS/0.2% (w/v) BSA (blocking buffer) at room temperature. Next, wells were coated with 100 ng ErbB3-IgG for 2 h in PBS/0.2% BSA/0.05% Tween20 (binding buffer). Wells were rinsed twice and incubated with 1-3 $\times 10^{10}$ $T1E^{46-50}$ phages in 0.1 ml binding buffer. After incubation for 2 h, unbound phages were removed by rinsing 12 times with washing buffer. Bound phages were eluted by addition of 0.1 ml glycine buffer (50 mM glycine/150 mM NaCl pH 2.7) for 10 min, and the eluate was neutralised with 25 µl of 1 M Tris/HCl pH 8.0. The elutate was used for phage titration and infection of logarithmic cultures of TG-1 cells.

Whole cell phage selection

Phage selections on MDA-MB-453 cells were carried out by incubation of 5-10 $\times$ 10^6 cells in suspension with 1-3 $\times$ 10^{10} tu $T1E^{46-50}$ phages in 3

ml blocking buffer. In the case of ErbB3-IgG depletion, the phages were subjected to ErbB3-IgG as described above prior to cell selection. The unbound phages from ErbB3-IgG wells were subsequently transferred to 12 ml Falcon tubes, diluted to 3 ml with binding buffer and added to MDA-MB-453 cells. After incubation for 2 h on a rowing boat shaker, cells were rinsed seven times in washing buffer by spinning for 4 min at 1000 rpm, followed by resuspension and a final wash with PBS. Cells were transferred twice to clean tubes to remove phages non-specifically bound to the plastic. Specific elution was performed by competition with a 1:1 mixture of the anti-ErbB2 monoclonal antibodies L26 and L96 (Neomarkers, Fremont, CA (34)) at 50 µg/ml in binding buffer for 1 h at 4°C on a rowing boat shaker. Cells were spun and the supernatant was collected as ErbB2/ErbB3 phage fraction. The remaining cell-bound phages were harvested by a 10 min incubation in 1 ml acid elution buffer, followed by neutralisation upon adding 0.2 ml 1 M Tris-HCl pH 8.0. Cells were spun for 5 min at 1200 rpm, and the eluate was collected as ErbB-3 fraction. The distinct eluted fractions were used for phage titration and infection of logarithmic cultures of TG-1 cells.

Phage ELISA

Small scale phage preparations were used for the screening of individual clones in ELISA assays. PEG 8000 precipitates of culture broths of clones grown overnight in 2 ml 2xTY supplemented with 12.5 µg/ml tetracyclin in 12-wells plates were resuspended in 100 µl PBS, typically resulting in phage titers of 1-5 × 10^{10} tu/ml. Whole cell ELISAs on 32D sublines in suspension were performed as described (29). The procedure for ErbB-IgG ELISA was similar as described (20), with the following modifications: homodimeric ErbB3-IgG fusion proteins were coated at 50 ng/well, and heterodimeric ErbB2/3-IgG at 100 ng/well in 0.1 ml of binding buffer.

Cell proliferation assays

32D cells that coexpress ErbB2 and ErbB3 (D23 cells) were washed in RPMI 1640 medium to deprive them of IL-3. Subsequently cells were seeded into 96-well tissue culture plates at a density of 5.0 × 10^4 cells/well in 0.1 ml RPMI supplemented with 0.1% BSA, together with serial dilutions of filter-sterilised phages or recombinant growth factors. Cell survival was determined after 24 hours of incubation at 37 °C using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) assay, as previously described (29).

ACKNOWLEDGMENTS

We thank Y. Yarden and D. Harrari (Weizmann Institute of Science, Rohovot, Israel) for the kind gift of the 32D cells and gene constructs encoding the ErbB-IgG fusion proteins. The NRG1β precursor was generously supplied by Genentech Inc. (San Francisco, CA). The present work was supported by grants from the Dutch Cancer Society and the Netherlands Organisation for Advancement of Research.

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Negative constraints underlie the ErbB specificity of epidermal growth factor-like ligands

Journal of Biological Chemistry 281, 40033-40040 (2006)

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ABSTRACT

Epidermal growth factor (EGF)-like growth factors bind their ErbB receptors in a highly selective manner, but the molecular basis for this specificity is poorly understood. We have previously shown that certain residues in human EGF(Ser2-Asp3) and TGFα (Glu26) are not essential for their binding to ErbB1 but prevent binding to ErbB3 and ErbB4. In the present study, we have used a phage display approach to affinity-optimize the C-terminal linear region of EGF-like growth factors for binding to each ErbB receptor and thereby shown that Arg45 in EGF impairs binding to both ErbB3 and ErbB4. By omitting all these so-called negative constraints from EGF, we designed a ligand designated panerbin that binds ErbB1, ErbB3, and ErbB4 with similarly high affinity as their wild-type ligands. Homology models, based on the known crystal structure of TGFα-bound ErbB1, showed that panerbin is able to bind ErbB1, ErbB3, and ErbB4 in a highly similar manner with respect to position and number of interaction sites. Upon *in silico* introduction of the experimentally known negative constraints into panerbin, we found that Arg45 induced local charge repulsion and Glu26 induced steric hindrance in a receptor-specific manner, whereas Ser2-Asp3 impaired binding due to a disordered conformation. Furthermore, radiolabeled panerbin was used to quantify the level of all three receptors on human breast cancer cells in a single radio receptor assay. It is concluded that the ErbB specificity of EGF-like growth factors primarily results from the presence of a limited number of residues that impair the unintended interaction with other ErbB receptors.

INTRODUCTION

The human genome encodes four ErbB tyrosine kinase receptors, designated ErbB1 (HER1 or EGFR receptor), ErbB2, ErbB3, and ErbB4. They are activated by a total of 11 different EGF-like growth factors, and in combination this set of receptors and ligands plays a crucial role in the growth control of mammalian cells and the development of multicellular organisms (1). Particularly, ErbB1 and ErbB2 are frequently overexpressed in many epithelial tumors, and as a consequence the ErbB signaling network is currently one of the main targets for the development of anti-tumor drugs (2–5).

ErbB receptors show a highly pronounced ligand specificity, but the molecular basis for this binding specificity is still poorly understood. ErbB1 binds seven distinct ligands (EGF, TGFα, amphiregulin (AR), epigen (EPGN), BTC, epiregulin (EREG), and heparin-binding EGF-like growth factor HB-EGF), ErbB3 binds only NRG1 (neuregulin 1) and NRG2, and ErbB4 interacts with NRG1, NRG2, NRG3, NRG4, BTC, epiregulin, and heparin-binding EGF-like growth factor. ErbB2 does not bind ligand but is the preferred

heterodimerization partner of all liganded ErbB receptors. It has so far been anticipated that the ErbB specificity of EGF-like growth factors results from the presence or absence of specific residues that are directly involved in receptor binding. However, a large number of site-directed mutagenesis studies, particularly on EGF, TGFα, and NRG1β, have identified numerous residues that upon mutation reduce the binding affinity for their respective ErbB receptor, but in general these mutations do not affect receptor specificity. This agrees with crystal structure data, which have shown that many residues in both EGF and TGFα interact directly with ErbB1 (6, 7). No such data are available yet for ErbB3 and ErbB4, since the relevant crystal structures are still lacking, but based on the strong homology of the different ErbB receptors and the high structural similarity between the various EGF-like growth factors, it seems unlikely that for binding to these receptors a fully different set of ligand residues is involved. Moreover, phylogenetic tree analysis has indicated that TGFα and BTC are closely related and may have arisen from a common ancestor gene (8), but still TGFα is an ErbB1-specific ligand, whereas BTC interacts with both ErbB1 and ErbB4. AR and HB-EGF are similarly related but also show distinct receptor specificity. This suggests that during evolution, specific strategies have been developed to obtain the desired receptor specificity.

Previous studies have indicated that the receptor binding specificity of EGF-like growth factors can be compromised by making chimeras of different ligands. Barbacci *et al.* (9) have shown that a chimera between EGF and NRG1β, designated biregulin, not only binds with high affinity to ErbB1 but also shows low affinity competition with NRG1β for receptor binding. Our previous studies (10) have shown that this is similarly true for various chimeras of the ErbB1-specific ligands EGF and TGFα. In particular, T1E, which is composed of EGF sequences with the N-terminal linear region of TGFα, is not only a high affinity ligand for ErbB1 but also a low affinity ligand for ErbB3 and ErbB4. Subsequent analysis showed that EGF is unable to bind ErbB3, because it lacks the His2-Phe3 sequence of TGFα in its N-terminal linear region, whereas TGFα is unable to bind ErbB3 because of the bulky, negatively charged Glu26 (EGF numbering) in the so-called B-loop (10). In contrast, T1E, which contains the His2-Phe3 sequence but lacks Glu26, is able to interact with ErbB3. Intriguingly, both crystal structure and mutational analysis revealed that these specific residues are not essential for high affinity binding to ErbB1. In a more recent study (11), we have further optimized the amino acid sequence of T1E in its N- and C-terminal linear region to derive a mutant, designated WVR/EGF/IADIQ, that binds ErbB1 with similarly high affinity as EGF and ErbB3 with similarly high affinity as NRG1β but shows only low affinity for ErbB4.

Based on the above observations, we have postulated that EGF-like growth factors do not only contain residues that are directly involved in ErbB binding (positive constraints) but in addition residues that are not essential for binding to their cognate receptor but that prevent their unintended

interaction with other ErbB receptors (negative constraints). In the present study, we provide evidence that such negative constraints in ligand molecules form the primary basis for their ErbB specificity. Using a phage display approach in which the C-terminal linear region of T1E has been randomized, we have established the optimal sequences for ligand binding to ErbB1, ErbB3, and ErbB4. Our data show that each of these three receptors has unique requirements for ligand binding, but that a consensus sequence can be derived that permits high affinity binding to all three receptors. From the data obtained, we conclude that T1E is a low affinity ligand for ErbB3 and ErbB4, because Arg45 forms a negative constraint for binding to ErbB3 and ErbB4 but not to ErbB1. Removal of this arginine from T1E resulted in the generation of a ligand, designated panerbin, that binds all three receptors with similarly high affinity as their wild-type ligands. Homology modeling studies showed that panerbin binds ErbB1, ErbB3, and ErbB4 in an almost identical manner, indicating that ErbB receptors recognize similar positive constraints in their ligand molecules. However, introduction of the identified negative constraints into panerbin resulted in local charge repulsion, conformational impairment, or steric hindrance for binding to ErbB3 and ErbB4 but not for ErbB1, thereby providing a molecular basis for their role in receptor specificity.

MATERIALS AND METHODS

Cell Lines

Interleukin 3-dependent murine 32D hematopoietic progenitor cells transfected with distinct human ErbB encoding plasmids were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and 0.25 ng/ml murine interleukin-3 (Promega, Madison, WI) and subsequently kept under continuous selection using 0.6 mg/ml G418 (Calbiochem). HER-14 cells and T47-14 cells were cultured in gelatinized flasks in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (12).

Selection of High Affinity T1E Variants on ErbB-Fc Fusion Proteins

Construction of the phage T1E (residues 44–48) library and the preparation of ErbB-Fc fusion proteins have been described previously (13). Immunoabsorbent 24-well plates were precoated overnight at 4 °C with 0.2 μg of goat anti-human Fc-specific IgG (Jackson Immunoresearch Laboratories, West Grove, PA) in 0.2 ml of PBS (137mM NaCl, 2.7mM KCl, 1 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Wells were washed with PBS plus 0.05% (v/v) Tween 20 and subsequently blocked with 0.2 ml of PBS plus 0.2% (w/v) BSA for 1 h at room temperature to prevent nonspecific binding. The wells were then

coated with 100 ng of ErbB-Fc for 2 h in PBSplus 0.2% BSA and 0.05% Tween 20 (binding buffer). Wells were subsequently washed twice with PBS/Tween and incubated with $3*10^8$ T1E (residues 44–48) phages in 0.1 ml of binding buffer. After incubation for 2 h, unbound phages were removed by rinsing the wells 12 times with PBS/Tween. In the first selection round, phages were eluted by adding 0.1 ml of glycine buffer (50 mM glycine, 150 mM NaCl, pH 2.7) for 10 min, and the eluate was neutralized by the addition of 25 μl of 1 M Tris/HCl, pH 8.0. In subsequent selection rounds, the bound phages were eluted specifically with 1 μg of murine EGF (for ErbB1) or 1 μg of NRG1β (for ErbB3 and ErbB4) in 1 ml of binding buffer for 1 h at room temperature. The eluate was used for phage titration and infection of logarithmic cultures of *Escherichia coli* TG-1 cells.

Phage Enzyme-linked Immunosorbent Assay on ErbB-Fc

Nunc immunoadsorbent 96-well plates were precoated overnight at 4°C with 0.2 μg of goat anti-human Fc-specific IgG (Jackson Immunoresearch Laboratories) in 100 μl. Wells were washed in PBS/Tween and blocked for 1 h in 0.2ml of PBS/BSA at room temperature. Next, wells were coated for 2 h with 100 ng of ErbB-Fc in binding buffer, subsequently washed twice, and incubated with phages in 0.1 ml of binding buffer. After incubation for 2 h, unbound phages were removed by rinsing three times with PBS/Tween. For detection of bound phages, wells were incubated for 1 h at room temperature with peroxidase-conjugated anti-M13 antibody (Amersham Biosciences) in PBS/BSA. To remove unbound phages, wells were washed three times with PBS. Subsequently, 0.1 ml of substrate, consisting of 0.4 μM 3,3'5,5'-tetramethyl-benzidine (Sigma) and 0.03% H_2O_2 in 0.11 M citrate buffer, pH 5.5, was added to the wells. The reaction was terminated after 5 min by the addition of 0.05 ml of 1 M H_2SO_4. Absorption was read at 450 nm.

Construction of T1E/YYDLL

Mutations in the C-terminal linear region were made using mutagenic oligonucleotide primers with pEZZ/FX T1E as template. Mutant gene products were subsequently introduced with the pEZZ vector using the BamHI/SalI sites, and the constructs were verified by automated cycle sequencing. Recombinant T1E/YYDLL was expressed as a protein A-tagged fusion protein in the protease K-deficient *E. coli* strain KS474. T1E/YYDLL was purified by affinity chromatography using IgG-Sepharose, followed by FactorX cleavage of the protein A tag, an additional round of affinity chromatography to remove the tag, and a final reverse-phase HPLC purification step (10). The amount of growth factor was calculated from the peak area (absorption at 229 nm) in the reverse-phase HPLC chromatogram, using murine EGF as a standard.

Radioactive Ligand Displacement Experiments

Natural murine EGF (Bioproducts for Science Inc., Indianapolis, IN) and recombinant hNRG1β residues 117–246 (R&D Systems, Minneapolis, MN) were iodinated enzymatically to a specific activity of 1.1 Ci/mol (14). Ligand binding displacement studies on HER-14, 32D3, and T47-14 cells were performed as described previously (13, 15). For differential binding competition analysis, $3.0x10^5$ MCF-7, CAMA-1, or T47D human breast cancer cells were seeded in 24-well plates in 1 ml of Dulbecco's modified Eagle's medium with 10% newborn calf serum. After 2 days, the confluent monolayers were incubated for 2 h on ice with 100μl of 10 ng/ml [^{125}I]T1E/YYDLL, radiolabeled as above, in the presence of serial dilutions of unlabeled T1E/YYDLL, TGFα, NRG1β, or BTC. Cells were subsequently washed twice with ice-cold PBS. Cells were lysed in 0.5ml of 1% Triton X-100, and the bound radioactivity was measured by γ-counting.

Homology Modeling of T1E/YYDLL in Complex with ErbB1, ErbB3, and ErbB4

Homology models were constructed for the extracellular domains of ErbB1, ErbB3, and ErbB4, all in complex with T1E/YYDLL. Initial models were built using both the ErbB1-EGF complex (7) and the ErbB1-TGFα complex (6, 16) as a template. Validation of the models with WHAT CHECK (17) identified template 1MOX, the ErbB1-TGFα complex solved at a resolution of 2.5 Å (6), as the best choice both for receptor and ligand. The amino acid side chains in the final models were positioned using SCWRL (17). Subsequently, the models were refined in YASARA using the Yamber2 force field and the associated protocol (18) until the WHAT IF (19) quality indicators (Ramachandran plot, backbone conformation, and three-dimensional packing quality) converged. Coordinate files of the final models are available from the authors upon request.

RESULTS

Selection of Optimal C-terminalSequences for ErbB1, ErbB3, or ErbB4 Binding

We have previously shown that T1E, obtained by introducing TGFα sequences into the N-terminal linear region of EGF, is a broad ErbB-activating ligand with high affinity for ErbB1 and low affinity for ErbB3 and ErbB4 (10). Additional studies have indicated that the affinity of T1E for ErbB3 can be further enhanced by altering the C-terminal linear region (11, 13). To identify C-terminal sequences that are optimal for binding of T1E to each of these

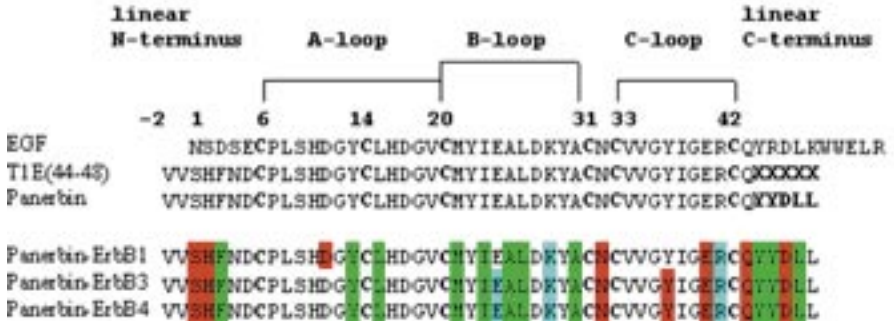

Figure 1. Sequences of EGF, the T1E (residues 44–48) library, and panerbin. The conserved cysteines, looped regions, and N- and C-terminal linear regions are indicated. Numbering throughout the study is according to hEGF. *X* represents randomized positions. *Colored* residues represent the sites of direct receptor interaction, according to homology models of panerbin with the various ErbB receptors. Residues in *red* are involved in hydrogen bond formation, residues in *green* are involved in hydrophobic interactions, and residues in *blue* are involved in salt bridge formation with the indicated ErbB receptors.

three ErbB receptors, we expressed T1E on phage whereby the DNA sequence encoding residues 44–48 was randomly mutated (residue numbering according to EGF; see Fig. 1 for sequences). To select ErbB-specific high affinity binders, the resulting phage library was incubated with Fc-ErbB fragments of ErbB1, ErbB3, and ErbB4, respectively, during three rounds of selection. ErbB2 with its permanent open conformation cannot bind ligand (16) and was therefore not included. Supplemental Table 1 summarizes the phage input/output ratios during the various selection rounds. Fig. 2 shows that in the case of ErbB1, a phage pool with similarly high affinity as T1E was already obtained after a single round of selection, whereas a phage pool with strongly enhanced affinity for both ErbB3 and ErbB4 compared with T1E was obtained after 2–3 rounds of selection. From each of the receptor-specific phage pools obtained after three rounds of selection, multiple clones were sequenced (see supplemental Table 2, *a*–*c*). T1E variants with distinct amino acid sequences were obtained, indicating that multiple C-terminal sequences facilitate ErbB-specific high affinity binding. Fig. 3, *a*–*c*, show the frequency distribution of individual amino acids in the selected variants. Based on these frequencies, consensus sequences (occurrence > 50%) for optimal receptor binding of T1E (residues 44–48) selectants could be assigned, as follows: for ErbB1, *XX*DL*X*; for ErbB3, I(F/Y)D(Ar/Hp)*X*(where Ar represents an aromatic and Hp represents a hydrophobic residue); and for ErbB4, YN(D/E)(L/V/I)*X*. These data show that within the context of a T1E background, all three ErbB receptors display distinct requirements for high affinity ligand binding.

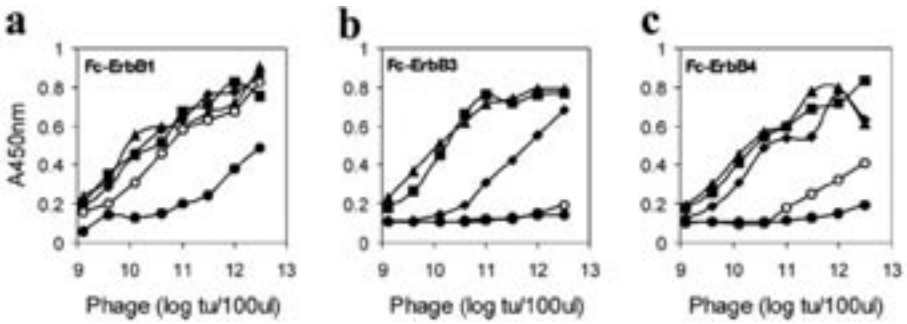

Figure 2. Binding characteristics of T1E (residues 44–48) phage pools after the indicated rounds of selection on Fc-ErbB-1 (*a*), Fc-ErbB3 (*b*), or Fc-ErbB4 (*c*). Phage pools were tested in a phage binding enzyme-linked immunosorbent assay. Phage T1E (open circles), native T1E (residues 44–48) library (closed circles), first round selection (closed diamond), second round selection (closed square), and third round selection (closed triangle). The phage input/output ratios of each selection round are shown in supplemental Table 1.

Selection of a Pan-ErbB Ligand

To test if the requirements for ligand binding to ErbB1, ErbB3, and ErbB4 are mutually exclusive, the phage pool obtained after two rounds of selection on Fc-ErbB3 was subsequently selected for high affinity binders to Fc-ErbB4 and Fc-ErbB1. Binding analysis indicated that the phage pool obtained after two selection rounds on ErbB3 already showed high affinity for both ErbB3 and ErbB1 but only intermediate affinity for ErbB4 (see supplemental Fig. 1). However, after an additional selection round on ErbB4, a phage pool with high affinity for all three ErbB receptors was obtained, although no deliberate selection on ErbB1 had been carried out. The amino acid sequences of the variants selected after binding to both ErbB3 and ErbB4 are listed in supplemental Table 2*d*, whereas the corresponding frequency distribution of residues at each position is presented in Fig. 3*d*. Based on these frequencies a consensus sequence of YYDL*X* was obtained for the C-terminal linear region of the T1E (residues 44–48) selectants that bind with high affinity to ErbB1, ErbB3, and ErbB4. This consensus sequence thus reflects a unique combination of residues for binding to all three receptors. Phage display mutational analysis will favor residues that provide a positive constraint for receptor binding, whereas negative constraints will be eliminated. According to the data presented in Fig. 3, residue Leu47 appears to be a major positive constraint in the linear C-terminal region for binding to ErbB1, Asp46 for ErbB3, and Tyr44 for ErbB4 (all > 80% occurrence). The main difference between T1E/YYDL*X* and T1E itself is the mutation R45Y, since the residue at position 48 appears less important according to the pan-ErbB consensus sequence. This suggests that Arg45 forms a negative constraint for binding to ErbB3 and ErbB4 but not for interaction with ErbB1.

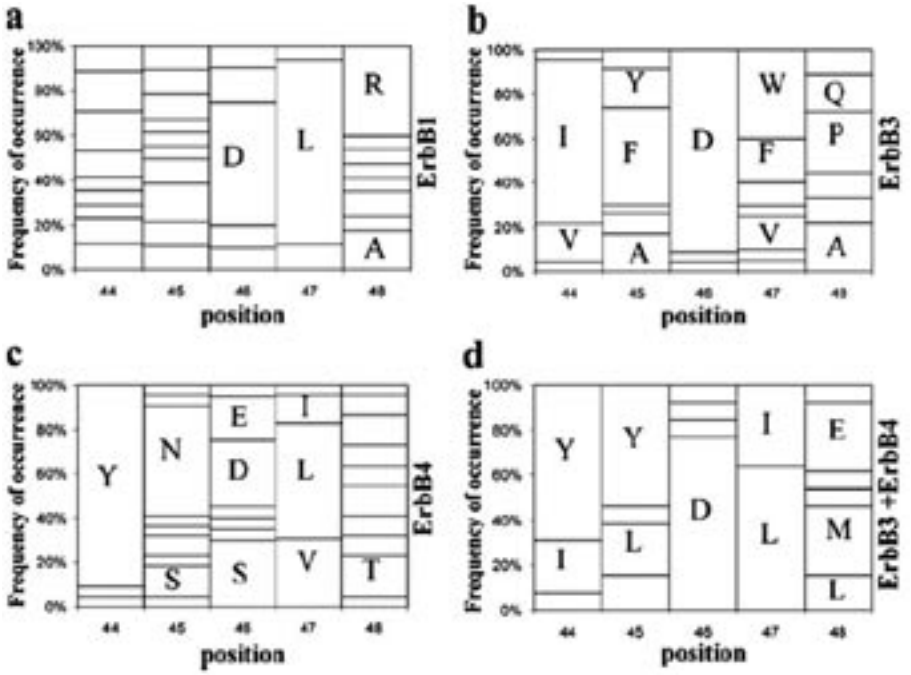

Figure 3. Schematic representation of the amino acid distribution of selected T1E (residues 44–48) variants after three rounds of selection on Fc-ErbB1 (*a*), Fc-ErbB3 (*b*), or Fc-ErbB4 (*c*) and the combination of two rounds on Fc-ErbB3 and one round on Fc-ErbB4 (*d*). Sequences of individual selectants are depicted in supplemental Table 2.

Characterization of the Recombinant Pan-ErbB Ligand T1E/YYDLL on Living Cells

The sequence T1E/YYDLL was found twice within the phage clones characterized, and since it fully corresponds with the obtained consensus sequence for a ligand that binds all three ErbB receptors, we subsequently tested T1E with YYDLL at positions 44–48 (see Fig. 1) as a purified recombinant protein for its ErbB affinity on living cells. Fig. 4*a* shows that recombinant T1E/YYDLL binds with similarly high affinity as T1E and EGF to ErbB1 on HER14 cells (IC50 = 40 ng/ml). On 32D cells transfected with ErbB3, the affinity of T1E/YYDLL (IC50 = 15 ng/ml) was increased 60-fold compared with T1E (IC50 = 900 ng/ml) and reached almost the same level as the endogenous ligand NRG1β (IC50 = 7 ng/ml). The binding affinity of T1E/ YYDLL (IC50 = 20 ng/ml) to ErbB4 on T47-14 cells was even higher than that of NRG1β (IC50 = 50 ng/ml). This shows that, despite the fact that ErbB1, ErbB3, and ErbB4 have distinct binding requirements, T1E/YYDLL can bind all three receptors, with an affinity for ErbB3 and ErbB4 similar to NRG1β and an affinity for

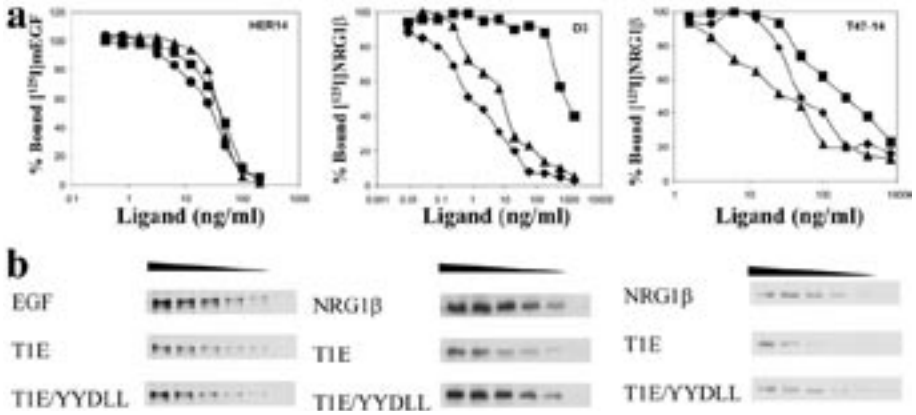

Figure 4. ErbB affinity of T1E/YYDLL. *a*, ligand binding displacement analysis of T1E/YYDLL on cells expressing distinct ErbB receptors. Displacement of ^{125}I-labeled murine EGF binding to HER-14 cells expressing ErbB1 (*left*), of [^{125}I]NRG1β binding toD3cells expressing ErbB3 (*middle*), and of [^{125}I]NRG1β binding to T47-14 cells expressing ErbB4 (*right*), respectively. EGF (closed circle), T1E (closed square), NRG1β (closed diamond), and T1E/YYDLL (closed triangle). Results are the mean of at least three independent experiments performed in duplicate. *b*, receptor tyrosine phosphorylation induced by T1E/YYDLL on HER14 cells (*left*), D23 cells expressing both ErbB2 and ErbB3 (*middle*), and T47-14 cells (*right*). Cells were stimulated with 2-fold serial dilutions of unlabeled growth factor starting from 25 ng/ml. Whole cell lysates were loaded onto SDS-polyacrylamide gels followed by immunoblotting with anti-phosphotyrosine antibodies. The 180-kDa band is shown.

ErbB1 similar to EGF. Fig. 4*b* shows that T1E/YYDLL has a similar ability as EGF and T1E to induce tyrosine phosphorylation of ErbB1. Since ErbB3 has a defective tyrosine kinase domain, the ability of T1E/YYDLL to induce ErbB3 phosphorylation was tested on 32D23 cells expressing both ErbB2 and ErbB3. The data show that T1E/YYDLL induced tyrosine phosphorylation of ErbB3 to the same extent as NRG1β, whereas T1E was much less active. On ErbB4, T1E/YYDLL was as least as potent as NRG1β in inducing receptor tyrosine phosphorylation, again much more so than T1E. Thus, T1E/YYDLL is a pan-ErbB ligand that is capable of high affinity binding to all three receptors and a potent activator of ErbB1, ErbB3, and ErbB4. We propose to designate this ligand panerbin, because it facilitates high affinity binding and activation of all three receptors.

Homology Modeling of T1E/YYDLL in Complex with ErbB1, ErbB3 and ErbB4 Explains the Pan-ErbB Character

The availability of a ligand that binds ErbB1, ErbB3, and ErbB4 with high affinity allows a structural comparison of three different receptors in

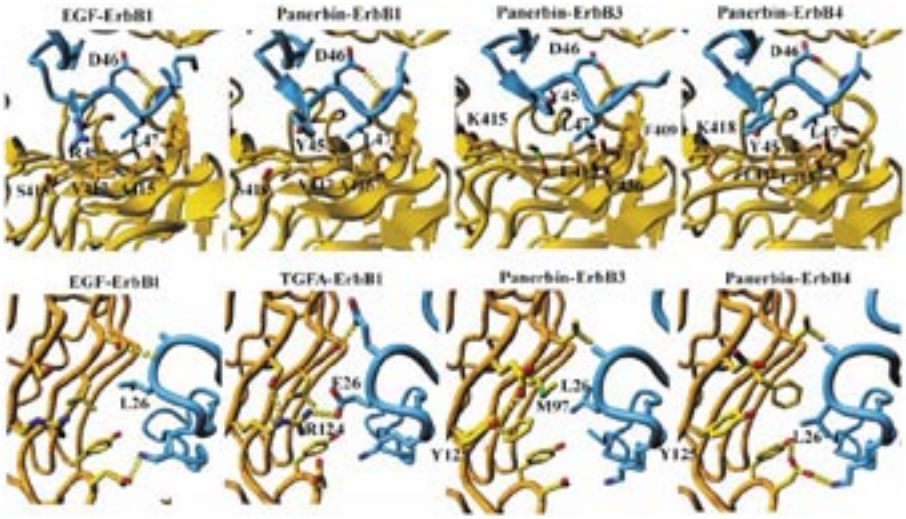

Figure 5. Homology models for the structure of EGF, TGFα, T1E, and panerbin in complex with the extracellular domain of ErbB1, ErbB3, and ErbB4. *Top*, *close-up pictures* showing *ribbon representations* of the B-loop of the ligand (*blue*) upon interaction with the hydrophobic pocket of the L1 domain of the indicated receptors (*yellow*). *Bottom*, interaction of the C-terminal linear region of the ligands with the hydrophobic pocket of the L2 domain of the receptors. The amino acid numbering of the ligands is according to that of EGF, and the amino acid numbering of the receptors is according to Jorrisen *et al.* (32).

complex with the same ligand. In order to do so, homology models were constructed for the receptor-ligand complexes, based on the published crystal structure of the ErbB1-TGFα complex. Crystal structures of ErbB3 and ErbB4 are only available in the unliganded, autoinhibited conformation. A comparison showed that the L1 and L2 domains of ErbB3 and ErbB4 had similar conformations in the obtained homology models as in the crystal structures of the autoinhibited conformation (data not shown). When considering the amino acids in T1E/YYDLL that directly interact with residues in the receptor, only minor differences were observed between ErbB1, ErbB3, and ErbB4 (see Fig. 1, *colored residues*). These differences mainly resulted from the lack of a hydrogen bridge or a salt bridge partner in the receptor. These results indicate that these three receptors bind T1E/YYDLL in a highly comparable manner, by providing similar positive constraints for binding. In order to understand the mechanisms whereby negative constraints in EGF-like growth factors induce ErbB specificity, we subsequently compared the existing crystal structures of EGF and TGFα in complex with ErbB1, with the homology models of T1E/YYDLL bound to ErbB1, ErbB3, and ErbB4. We particularly focused on the receptor environment of the ligand residues Arg45, Glu26, and Ser2-Asp3, which are negative constraints for binding to ErbB3 and ErbB4 but not to ErbB1. Fig. 5

(*top*) shows that in the ErbB1-EGF complex Arg45 of EGF comes within 4Å of Ser418 of ErbB1, whereas the corresponding Tyr45 of T1E/YYDLL comes close to Ser418 of ErbB1, Lys415 of ErbB3, and Lys418 of ErbB4. This implies that when Arg45 is reintroduced into T1E/YYDLL, it would result in considerable charge repulsion with the lysines in ErbB3 and ErbB4 but not with the serine in ErbB1. This most likely explains why T1E is a high affinity ligand for ErbB1 but only a low affinity ligand for ErbB3 and ErbB4. In fact, Tyr45 in T1E/YYDLL may well contribute to hydrophobic interactions and thereby enhance the binding affinity for ErbB3 and ErbB4 as a positive constraint. From mutant studies, it is known that Arg45 is not essential for EGF binding to ErbB1 (15), and therefore we conclude that Arg45 plays a major role in preventing binding of EGF to ErbB3 and ErbB4. Fig. 5 (*bottom*) shows that in the ErbB1-TGFα complex Glu26 of TGFα forms a salt bridge with Arg124 of ErbB1, whereas Leu26 of T1E/YYDLL can participate in hydrophobic interaction with all three receptors. However, upon introduction into T1E/YYDLL, Glu26 would give a steric clash with Met97 when complexed to ErbB3. In the case of ErbB4, no steric hindrance of Glu26 would take place with Phe97 of the receptor, although it cannot contribute to receptor binding in a positive manner as observed for Leu26. Crystal structure analysis has shown that the N-terminal linear region of EGF is disordered and does not interact with ErbB1, whereas in the case of TGFα residues His2 and Phe3 interact with the ErbB1 residues Tyr101 and Leu68, respectively (see Fig. 1). Mutational analysis has shown that this His2-Phe3 motif is essential for high affinity ligand binding to ErbB3 but not to ErbB1 (20). We conclude that the negative constraint Ser2-Asp3 in EGF impairs binding to ErbB3 and ErbB4, most likely because it does not permit the proper β-sheet formation in the N-terminal linear region that is essential for binding to ErbB3 and ErbB4.

Determination of the Relative Amount of ErbB Receptors on Breast Tumor Cells by a Differential Binding Competition Assay Using ^{125}I-Labeled Panerbin

Since panerbin binds with high affinity to ErbB1, ErbB3 and ErbB4, it can be applied to determine the relative amount of ErbB receptors on tumor cells in a single radioreceptor assay. The assay is based on binding of ^{125}I-labeled panerbin and subsequent competition by various unlabeled ligands with defined ErbB specificity.We applied this method to analyze the ErbB densities of T47D, MCF7, and CAMA-1 human breast tumor cell lines, which are known to express different sets of ErbB receptors. In the presence of nearly saturating concentrations of ^{125}I-labeled panerbin (10 ng/ml), unlabeled TGFα was used to compete for ErbB1-specific binding, BTC was used to compete for binding to ErbB1 and ErbB4, and NRG1β was used to compete for binding to ErbB3 and ErbB4. These receptor specificities were confirmed in a binding competition analysis of ^{125}I-panerbin by these ligands on cells with known ErbB composition (see supplemental Fig. 2). Based on the

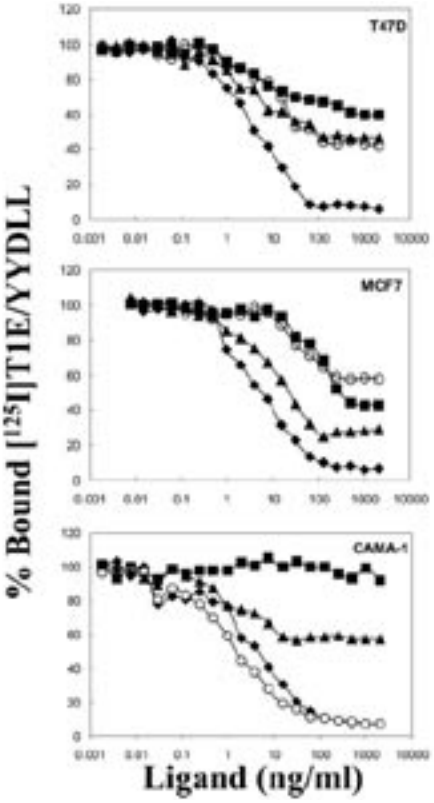

Figure 6. Determination of the ErbB status of human breast cancer cells. Binding competition assays were performed using 10 ng/ml [^{125}I]panerbin to occupy at least 50% of available ErbB receptors. Bound over added radioactivity was 4% (MCF7), 10% (T47D), and 10% (CAMA-1), corresponding to a total of $4.0x10^4$, $1.0x10^5$, and $1.0x10^5$ occupied ligand binding sites/cell, respectively. Serial dilutions of unlabeled T1E/YYDLL (closed diamond), TGFα (closed square), NRG1β (open circle), and BTC (closed triangle) were added to differentially compete for ErbB-selective binding (TGFα for ErbB1; BTC for ErbB1 and ErbB4; NRG1β for ErbB3 and ErbB4).

amount of ^{125}I-labeled panerbin bound and the differential competition curves presented in Fig. 6, we could calculate that T47D cells contain at least $4x10^4$ ligand binding receptors per cell, of which 41% could be attributed to ErbB1, 44% to ErbB3, and 15% to ErbB4; MCF7 cells contain a total of $1x10^5$ ligand binding receptors/cell, of which 48% were ErbB1, 40% were ErbB3, and 12% were ErbB4; and CAMA-1 cells contain $1x10^5$ ligand binding receptors/cell, of which 0% were ErbB1, 53% were ErbB3, and 47% were ErbB4. Together these data show that the designed EGF-like growth factor panerbin provides a new tool for direct analysis of ErbB binding levels in tumor cells, without the need of using multiple radiolabeled ligands.

DISCUSSION

EGF-like growth factors show a highly distinct receptor specificity. In the present study, we have shown that these growth factors not only contain residues that mediate high affinity binding to their cognate receptor (positive constraints) but in addition have residues that prevent the unintended binding to other ErbB receptors (negative constraints). Our results show that Arg45 in EGF is a major negative constraint for ligand binding to ErbB3 and ErbB4 but not to ErbB1. In previous studies, we had already identified Ser2-Asp3 in EGF and Glu26 in TGFα as negative constraints for binding to ErbB3 (10). By systematic release of these three negative constraints, we have now designed an EGF-based ligand, designated panerbin, that binds to ErbB3 and ErbB4 with similarly high affinity as NRG1β and to ErbB1 with similarly high affinity as EGF. Homology models show that panerbin can undergo similar interactions with ErbB1, ErbB3, and ErbB4. Moreover, the models provide an explanation for the observed ligand specificity of ErbB receptors on the basis of negative constraints.

The homology models indicate that at least 19 of the 55 residues of panerbin directly interact with not only ErbB1, but also ErbB3 and ErbB4, by a combination of electrostatic, hydrogen bond, and hydrophobic interaction (Fig. 1). This indicates that the positive constraints for binding of panerbin to these three receptors are highly similar. However, when the negative constraint Arg45 is introduced into panerbin (corresponding to T1E), a strong charge repulsion with Lys415 and Lys418 is predicted by the model for ErbB3 and ErbB4, respectively, but not for ErbB1 (Fig. 5, *top*). These homology models also showed that introduction of Glu26 into panerbin will result in a steric clash with Met97 of ErbB3, but not with Phe97 of ErbB4, whereas it can form a salt bridge with Arg125 of ErbB1. This agrees with the observation that Glu26 is conserved in the ErbB1- and ErbB4-specific ligand BTC. Finally, introduction of Ser2-Asp3 into panerbin will disorder the structure of the N-terminal linear region, which is known to affect binding to ErbB3 but not to ErbB1. It can therefore be concluded that the identified three negative constraints act as ErbB selectivity determinants, particularly for ErbB3. For ErbB4 only Arg45 has been identified experimentally as a negative constraint, whereas for ErbB1 binding no negative constraints have been identified yet. Interestingly, Hobbs *et al.* (21) have shown that NRG2β, which contains a phenylalanine at position 45, binds ErbB4 with high affinity, whereas its splice variant NRG2α shows only low affinity for this receptor, because it contains a lysine at this position. Based on a homology model, these authors (22) conclude that Phe45 contributes positively to ErbB4 binding by hydrophobic interaction with Lys438 (Lys418 in our numbering) of the receptor and that this positive constraint is lacking in NRG2α. According to our analysis, however, Lys45 in NRG2α will also induce charge repulsion with Lys418, thereby providing a negative constraint for ErbB4 binding.

Also from an evolutionary point of view, the ErbB signaling network

is of primary interest. Invertebrates, such as *Caenorhabditis elegans* (23) and *Drosophila melanogaster* (24), have only a single EGF receptor gene, regulated by one or multiple EGF-like growth factors (25). In urochordates, two distinct receptor genes have been identified, which have further evolved into four ErbB genes in vertebrates. During this evolutionary process, selection strategies must have been followed to obtain multiple ligands with distinct receptor specificity. Here we propose that the introduction of negative constraints has played a major role during this process of ligand diversification.

Using panerbin as a radiolabeled ligand, we have been able to characterize the ErbB expression profile of a number of ErbB overexpressing human breast tumor cell lines by a single radioreceptor assay. The present assay uses relatively high concentrations (10 ng/ml) of ^{125}I-panerbin to nearly saturate all ErbB receptors, which allows an estimation of the total and relative number of ligand binding ErbB receptors present. The assay does not give information, however, on cellular ErbB2 expression levels. When comparing with other techniques, Western blotting can only be used to compare the density of a specific receptor between different cells and not to determine the density of different receptors on the same cell. Moreover, not all ErbB receptors present in a cell will be able to bind an externally added ligand, either because the receptors are present in intracellular organelles or because they are present in a conformation that does not allow high affinity ligand binding. Cells can only respond to ligand molecules that bind plasma membrane receptors, and therefore the number of ligand binding receptors, as determined in the present radioreceptor assay, is the best parameter to correlate with the cell's responsiveness. Finally, quantitative PCR is able to determine absolute expression levels for different receptors, but only at the mRNA level.

The absolute and relative number of ErbB receptors are not only important when evaluating anti-tumor drugs designed to target these receptors but are also important for diagnosis and prognosis. Overexpression of ErbB1, often in combination with continuous secretion of TGFα and overexpression of ErbB2, is frequently observed in estrogen-independent carcinomas and has been associated with poor prognosis and poor response to chemotherapy (3). The clinical significance of ErbB3 and ErbB4 in tumor formation is less clear, but there is increasing evidence that overexpression of ErbB3 in combination with ErbB2 is associated with tumor growth of the breast (26) and the bladder (27). The role of ErbB4 in tumorigenesis is less clear, because of the distinct role of the various isoforms of this receptor. In a recent publication, it has been shown that the ErbB4 isoform, which is cleavable by TACE and γ-secretase but lacks the phosphatidylinositol 3-kinase binding motif, is able to promote proliferation of breast cancer cells (28). It should be realized that the present binding assay only identifies the fraction of uncleaved ErbB4 receptors.

The therapeutic potential of EGF-like growth factors has been

demonstrated in wound healing (29), improvement of cardiac function, and survival in animals with ischemic, dilated, and viral cardiomyopathy (30) and reduction of ischemic-induced brain damage in rats (31). Clinical application of EGF-like growth factors, however, may require enhanced receptor selectivity particularly to discriminate between the effects mediated by ErbB3 and ErbB4. We have shown in this study that upon systematic release of negative constraints from EGF, a pan-ErbB ligand can be designed that binds ErbB1, ErbB3, and ErbB4 with wild type affinity. It will be a further challenge to identify additional negative constraints, particularly for ErbB1 and ErbB4, which can then be introduced in a selective manner to design ligands with any requested ErbB specificity and affinity.

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Supporting information

Table 1. Overview of phage selections on Fc-ErbB with the T1E(44-48) library*.

Fc-ErbB1			
Round	Input	Output	In/Out
1	$3.3x10^8$	$4.5x10^5$	722
2	$2.0x10^7$	$8.7x10^5$	23
3	$3.8x10^7$	$2.7x10^6$	14
Fc-ErbB3			
Round	Input	Output	In/Out
1	$3.3x10^8$	$3.0x10^4$	10,800
2	$2.5x10^7$	$2.2x10^4$	1136
3	$4.2x10^7$	$1.1x10^6$	38
4	$2.9x10^7$	$3.0x10^5$	97
Fc-ErbB4			
Round	Input	Output	In/Out
1	$3.0x10^8$	$1.2x10^5$	1,000
2	$2.0x10^7$	$4.2x10^5$	48
3	$4.0x10^8$	$4.5x10^7$	9
2 x ErbB 3, 1x ErbB4	$2.6x10^7$	$6.8x10^4$	381

* For each selection round the input and output number of phages is indicated in titrating units.

Table 2a. Amino acid sequences encoded by individual phage clones from the T1E(44-48) library after three rounds of selection for ErbB1 binding*.

T1E(44-48)	46	47	48	49	50
wt	Tyr	Arg	Asp	Leu	Lys
1	Asn	Leu	Asp	Leu	Pro
2	Phe	Leu	Glu	Met	Trp
3	Thr	Lys	Asp	Leu	Arg
4	Asn	Lys	Asp	Leu	Arg
5	Met	Arg	Thr	Leu	Asp
6	Gln	Arg	Asp	Leu	Met
7	Leu	Gln	Asp	Leu	Ala
8	Gln	Val	Arg	Val	Arg
9	Thr	Tyr	Asp	Leu	Arg
10	Lys	Thr	Glu	Leu	Arg
11	Leu	Gln	Asp	Leu	Val
12	Lys	Met	Thr	Leu	Ala
13	His	Ile	Glu	Leu	Glu
14	Val	Ile	Asp	Val	Arg
15	Trp	Glu	Arg	Leu	Thr
16	His	Leu	Asp	Leu	Ala
17	Glu	Val	Asp	Leu	Met
18	His	Thr	Asp	Leu	Arg
Consensus	X	X	Asp	Leu	X

* The consensus sequence represents the amino acids that are present in more than 40% of the selected clones. X=random.

Table 2b. Amino acid sequences encoded by individual phage clones from the T1E(44-48) library after three rounds of selection for ErbB3 binding*.

T1E(44-48)	46	47	48	49	50
Wt	Tyr	Arg	Asp	Leu	Lys
1	Val	Ala	Asp	Phe	Met
2	Ile	Phe	Asp	Trp	Met
3	Ile	Phe	Asp	Trp	Gln
4	Tyr	Tyr	Asp	Ile	Met
5	Ile	Trp	Asp	Phe	Pro
6	Ile	Tyr	Asp	Trp	Pro
7	Ile	Phe	Asp	Trp	Ala
8	Ile	Ala	Asp	Val	Gly
9	Ile	Ala	Asp	Ile	Gln
10	Ile	Ser	Thr	Ile	Arg
11	Val	Phe	Asp	Val	Gln
12	Ile	Val	Asp	Val	Gly
13	Val	Trp	Asp	Phe	Pro
14	Thr	Phe	Asp	Ala	Phe
15	Val	Tyr	Asp	Phe	Pro
16	Ile	Tyr	Asp	Trp	Pro
17	Ile	Phe	Asp	Trp	Ala
18	Ile	Phe	Asp	Trp	Ala
19	Ile	Phe	Asp	Trp	Ala
20	Ile	Ser	Ile	Gly	Arg
21	Ile	Phe	Asp	Trp	Ala
22	Ile	Ala	Asp	Leu	Pro
23	Ile	Phe	Asp	Trp	Ala
Consensus	Ile	Tyr/Phe	Asp	Ar/ Hp	X

* The consensus sequence represents the amino acids that are present in more than 40% of the selected clones. Ar = aromatic; Hp = hydrophobic.

Table 2c. Amino acid sequences encoded by individual phage clones from the T1E(44-48) library after three rounds of selection for ErbB4 binding*.

T1E(44-48)	46	47	48	49	50
Wt	Tyr	Arg	Asp	Leu	Lys
1	Tyr	Ala	Ser	Val	Asp
2	Met	Tyr	Leu	Pro	Glu
3	Tyr	Asn	Glu	Leu	Asn
4	Tyr	Ser	Asp	Val	Arg
5	Tyr	Leu	Ser	Ile	Thr
6	Tyr	Asn	Glu	Leu	Leu
7	Tyr	Asn	Asp	Leu	Asn
8	Tyr	Asn	Tyr	Leu	Pro
9	Ala	His	Asp	Ili	Ili
10	Tyr	Asn	Thr	Leu	Asn
11	Tyr	Asn	Ser	Leu	Ala
12	Tyr	Thr	Ser	Leu	His
13	Tyr	Ser	Ser	Leu	Asp
14	Tyr	Met	Glu	Val	Pro
15	Tyr	Asn	Asp	Leu	His
16	Tyr	Gln	Gln	Val	Thr
17	Tyr	Asn	Thr	Ile	Ile
18	Tyr	Ile	Asp	Val	Leu
19	Tyr	Ser	Ser	Leu	Trp
20	Tyr	Asn	Gln	Val	Thr
21	Tyr	Asn	Met	Leu	Pro
22	Tyr	Asn	Glu	Leu	Glu
23	Tyr	Leu	Asp	Val	Thr
Consensus	Tyr	Asn	Asp/Glu	Leu/Val/Ile	X

* The consensus sequence represents the amino acids that are present in more than 40% of the selected clones.

Table 2d. Amino acid sequences encoded by individual phage clones from the T1E(44-48) library after two rounds of selection for ErbB3 binding and one subsequent round of selection for ErbB4 binding*.

T1E(44-48)	46	47	48	49	50
Wt	Tyr	Arg	Asp	Leu	Lys
1	Tyr	Leu	Glu	Leu	Glu
2	Tyr	Tyr	Lys	Leu	Phe
3	Tyr	Leu	Gln	Leu	Glu
4	Tyr	Leu	Asp	Leu	Glu
5	Tyr	Tyr	Asp	Leu	Tyr
6	Tyr	Tyr	Asp	Leu	Leu
7	Ala	Tyr	Asp	Ile	Pro
8	Ile	Ala	Asp	Ile	Gln
9	Ile	Ala	Asp	Leu	Met
10	Ile	Phe	Asp	Leu	Glu
11	Tyr	Tyr	Asp	Ile	Met
12	Tyr	Tyr	Asp	Leu	Leu
13	Tyr	Tyr	Asp	Ile	Met
14	Glu	Leu	Asn	Ile	Trp
15	Ile	Val	Asp	Ile	Met
16	Glu	Leu	Gln	Leu	Glu
17	Tyr	Tyr	Asn	Ile	Met
19	Tyr	Leu	Thr	Ile	Asp
20	Ile	Ala	Asp	Val	Gly
21	Trp	Leu	Asp	Ile	Asp
Consensus	Tyr	Tyr	Asp	Leu	X

* Individual phage clones obtained after two rounds of selection on ErbB3 and one on ErbB4, were tested for binding affinity to ErbB1, ErbB3 and ErbB4. Sequences of variants that showed high binding affinity to all three receptors are indicated in white, others in gray. The consensus sequence represents the amino acids that are present in more than 40% of the selected clones indicated in white.

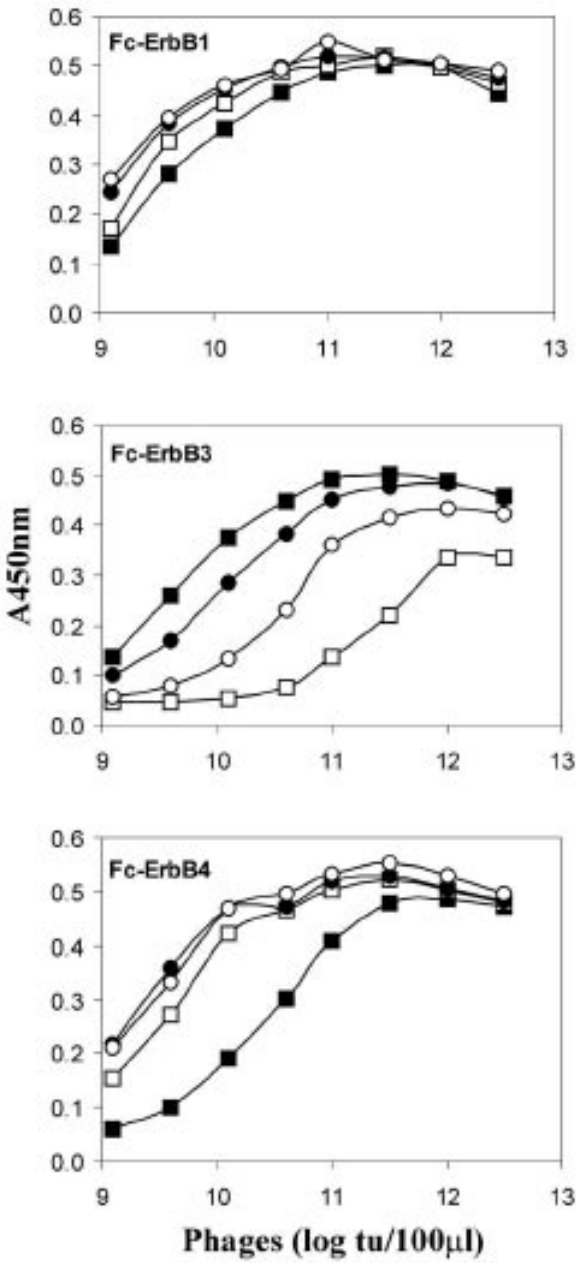

Figure 1.
Binding characteristics of T1E(44-48) phage pool after the 2nd round of selection on ErbB3 and ErbB4 and the subsequent ErbB3-ErbB4 cross selection. Second round ErbB3 (—■—), second round ErbB4 (—□—) and phage pool selected for two rounds on ErbB3 (—○—) and for one round on ErbB4 (—●—).

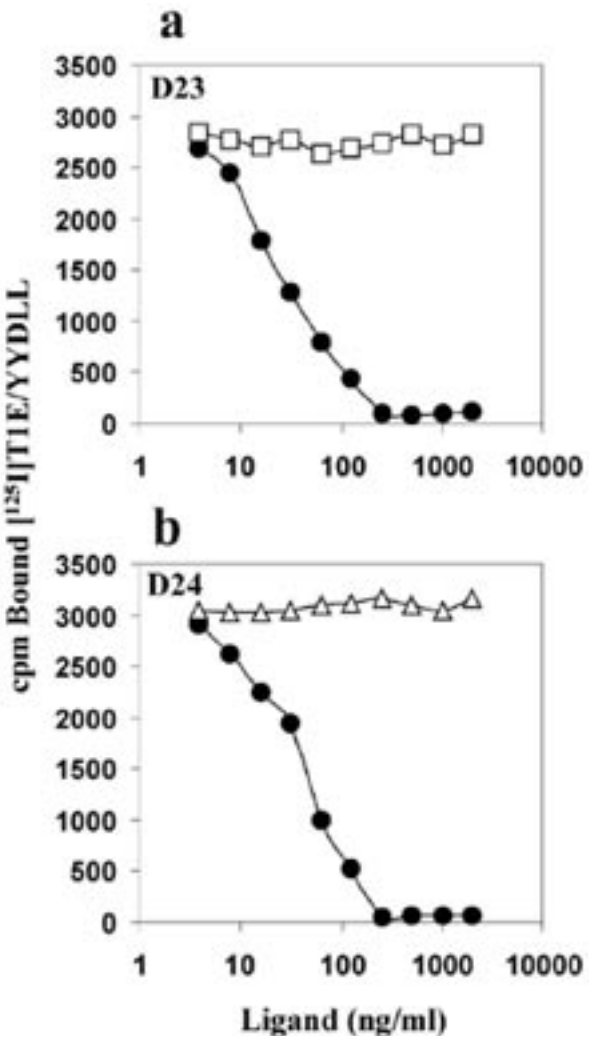

Figure 2 a. Displacement of 10 ng/ml [^{125}I]T1E/YYDLL by NRG1β (—●—) and BTC (—□—) on D23 cells expressing ErbB2 and ErbB3, showing that BTC at the concentrations used is unable to bind ErbB3. b: Displacement of 10 ng/ml [^{125}I]-T1E/YYDLL by NRG1β (—●—) and TGFA (—△—) on D24 cells expressing ErbB2 and ErbB4, showing that TGFA at the concentrations used is unable to bind ErbB4. For determining the ligand specificity of ErbB3 and ErbB4, cells were used that coexpress ErbB2, since this is known to enhance the affinity of the competing ligands.

4

Epidermal growth factor mutant with wild-type affinity for both ErbB1 and ErbB3

Biochemistry, 45, 4703-4710 (2006)

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ABSTRACT

The family of epidermal growth factor (EGF)-like ligands binds to ErbB receptors in a highly selective manner. Previous studies indicated that both linear regions of the ligand play a major role in determining receptor selectivity, and phage display studies showed that each region could be optimized independently for enhanced affinity. In this study we broadened the ErbB binding specificity of EGF by introducing the optimal sequence requirements for ErbB3 binding in both the N- and C-terminal linear region. One such EGF mutant, designated WVR/EGF/IADIQ, gained high affinity for ErbB3 and showed concomitant ErbB3 activation through ErbB2/ErbB3 heterodimers similar to the natural ErbB3 ligand NRG1β, while the capacity to bind and activate ErbB1 was fully maintained. Despite its high affinity for ErbB1 and ErbB3, this mutant was unable to activate ErbB1/ErbB3 heterodimers, as shown by cell survival and receptor phosphorylation analysis. It is concluded that, despite the fact that no naturally occurring ligand exists with this dual-specificity, high affinity binding to both ErbB1 and ErbB3 is not mutually exclusive. This mutant can be useful in a direct structural comparison of the ligand binding characteristics of ErbB1 and ErbB3.

INTRODUCTION

The ErbB signaling network consists of a family of peptide ligands that bind and activate the ErbB tyrosine kinase receptors ErbB1 (or EGFR), ErbB2 (or HER2/Neu), ErbB3 (or HER3), and ErbB4 (or HER4). Ligand binding is followed by receptor homo- or heterodimerization, receptor tyrosine phosphorylation, and the subsequent recruitment of cytoplasmic molecules, thereby initiating a cascade of signaling events (1, 2). The resulting biological responses play an important role in the proliferation and differentiation of almost all cell types in development. Overexpression of both receptors and ligands has been found particularly in epithelial cancers (3) and as a result the ErbB signaling network is currently one of the main targets in the development of anti-tumor drugs (4, 5).

Ligand binding is highly receptor specific and can be categorized into three distinct groups. A first group of ligands, composed of epidermal growth factor (EGF), transforming growth factor-α (TGFα), amphiregulin (AR), and epigen (EPG), binds only ErbB1. A second group is composed of the neuregulins (NRG) with their multiple isoforms, of which NRG1 and NRG2 bind both ErbB3 and ErbB4, while NRG3 and NRG4 bind exclusively ErbB4. A third group, consisting of epiregulin (EPR), betacellulin (BTC), and heparin-binding EGF (HB-EGF), binds both ErbB1 and ErbB4 (6, 7).

The observation that no natural ligand exists that binds both ErbB1 and ErbB3, suggests that these two receptors have diverged most during evolution in their ligand binding requirements. Important information on the

interaction of EGF-like growth factors with ErbB receptors and the mechanism of ErbB dimerization has been derived from crystallographic studies on the extracellular domain of ErbB1 and ErbB3 (8-11). In the absence of ligand, ErbB3 shows an auto-inhibited conformation maintained by interaction between subdomains II and IV. Upon ligand binding to both subdomains I and III the receptor conformation changes and the interaction between subdomains II and IV is released, as a result of which subdomain II becomes available for dimerization with another receptor. In the ligand-bound form of ErbB1, the B-loop region of EGF and TGFα, and in the case of TGFα also the linear N-terminus, interacts with subdomain I, whereas residues in the A-, C-loop and linear C-terminus are in close contact with subdomain III. Mutagenesis studies have indicated that particularly residues that interact with subdomain III are essential for high affinity binding to ErbB1 (12-14). In contrast, studies on NRG1β have shown that hydrophobic residues in the linear N-terminus and B-loop region are important for binding to ErbB3 (15-17). Assuming that all EGF-like ligands bind their respective ErbB receptor in a similar orientation, this suggests that in the case of ErbB3 particularly binding to subdomain I is essential for high affinity binding. This hypothesis is supported by the observation that NRG1β can bind to a soluble fragment of ErbB3, which contains subdomain I but not subdomain III (18). Thus, EGF-like ligands comprise multiple binding domains for interaction with either domain I or domain III of their respective receptor, and the relative contribution of each interaction may vary between different receptor-ligand combinations.

Previous work has indicated that mutant forms of EGF are not only able to bind ErbB1, but also ErbB3. In particular the chimeras T1E and biregulin, in which the N-terminus of EGF has been replaced by TGFα or NRG residues, respectively, exert high affinity for cells containing both ErbB2 and ErbB3, although they show only low affinity for ErbB3 alone (19, 20). Subsequently, we have used a phage display strategy to optimize the binding affinity of EGF-like growth factors for ErbB3. In the linear N-terminal region of EGF enhanced binding affinity for ErbB3 could be obtained by exchanging the wild-type sequence S2/D3/S4 into W2/V3/R4 (21). Within the C-terminal region of T1E, the binding affinity for ErbB3 could be strongly enhanced by introducing the sequence IFDWA or IADIQ at positions 44-48, instead of the wild-type sequence YRDLK (22). Thus these phage-bound variants differed from T1E in that they exerted also high affinity to ErbB3 in the absence of ErbB2.

In the present study we have combined the optimal sequences from the phage display studies in order to construct a recombinant EGF mutant with dual-specific high affinity for both ErbB1 and ErbB3. Our results show that particularly WVR(2-4)/EGF/IADIQ(44-48) competes not only effectively with EGF for binding to ErbB1, but also with NRG1β for binding to ErbB3. This mutant is a strong activator of ErbB1 homodimers and of ErbB2-ErbB3 heterodimers, but unlike NRG1β it is unable to activate ErbB1-ErbB3 heterodimers in cells that coexpress ErbB1 and ErbB3, most likely because

it preferentially induces the formation of ErbB1 homodimers. Despite a low affinity for ErbB4, this mutant is also able to induce growth stimulation of cells containing this receptor. These results indicate that linear N- and C-terminal region of EGF-like growth factors cooperate to direct ErbB receptor binding specificity.

MATERIALS AND METHODS

Cell lines and cell culture

T47-14 (a gift of M. H. Kraus, Birmingham, USA) and HER14 cells were cultured in gelatin-coated flasks in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% newborn calf serum (NCS). Interleukin (IL)-3 dependent murine 32D hematopoietic progenitor cells transfected with distinct human ErbB-encoding viral vectors or plasmids (a gift of Y. Yarden, Rehovot, Israel) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and 0.5 ng/ml murine IL-3 (R&D Systems). The 32D sublines used, designated D3 (containing 1.1 x 10^4 ErbB3 receptors/cell), D13 (containing 4.8 x 10^4 ErbB1 receptors/cell and 1.1 x 10^4 ErbB3 receptors/cell), D23 (containing ErbB2 and 1.3 x 10^4 ErbB3 receptors/cell), and D24 (containing ErbB2 and ErbB4 receptors) were kept under continuous selection using 0.6 mg/ml G418 (Invitrogen) and, in the case of D13 and D23 cells, in addition 0.4 mg/ml hygromycin B (Invitrogen) (23).

DNA constructs

Recombinant mutants of human EGF were constructed by PCR techniques using a gene construct encoding synthetic human EGF[1-53] as a template (24). The gene was linked at the 5' end to an IEGR encoding peptide sequence corresponding to the recognition sequence for the proteolytic enzyme factor Xα and subsequently to a sequence encoding two synthetic protein A-derived IgG-binding domains (Z-domains) by cloning into the expression vector pEZZ18 (Pharmacia) (25). The exact DNA sequence was verified by cycle sequencing (Perkin-Elmer).

A gene construct encoding the NRG1β EGF-like domain from serine 177 to serine 228 (a gift from Genentech Inc., San Francisco, CA) was used as a template to construct a NRG1β mutant optimized for binding to ErbB3 by means of splice overlap extension (SOE)-PCR (26). This mutant, here referred to as NRG1b-opt (for sequence see Figure 1), is also known in the literature as HRG-58 (17). Primers containing the desired mutations were obtained from Eurogentec. After cloning into the pCR2.1 vector (Invitrogen) and sequencing (Perkin Elmer), the DNA construct was ligated as an XhoI-SalI fragment into

the expression vector pPICZαA (Invitrogen). Subsequently, the expression vector was transformed into E.coli XL-2 Blue cells, selected on Zeocin and sequenced.

Expression and purification of growth factors

Recombinant mutant EGF-like growth factors were expressed as protein A-tagged fusion proteins in the proteinase K-deficient *E.coli* strain KS474 and isolated from the periplasmic fraction, as described (25). Briefly, growth factors were isolated by affinity chromatography using IgG-sepharose, followed by factor X cleavage of the protein A-tag, an additional round of affinity chromatography to remove the tag, and a final reverse phase (RP)-HPLC purification step. The amount of growth factor was calculated from the peak area (absorption at 229 nm) in the RP-HPLC chromatogram, using natural mEGF as a standard.

Recombinant NRG1b-opt was expressed in *Pichia pastoris* as described previously (27). In brief, expression of recombinant NRG1β-opt was induced by growing a selected Mut+ clone on BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 0.04% biotin, and 0.5% methanol) for four days at 30ºC with additional supplies of methanol every day. NRG1β-opt was purified from the collected culture medium by cation exchange chromatography (SP-550, Tosoh Biosciences). After dialysis against 0.5 M HAc, a final RP-HPLC purification step was performed (27).

Ligand binding displacement experiments

Natural mEGF (Bioproducts for Science Inc.), recombinant NRG1β-opt and WVR/EGF/IADIQ were radiolabeled using the Iodogen method (Pierce) according to the manufacturer's protocol for indirect labeling. NRG1b-opt was used in ligand binding displacement experiments on cells expressing ErbB3 or ErbB4, because this high affinity human NRG mutant is much more stable as radiolabeled ligand than the natural NRG1β, most likely because of the mutation of a methionine into isoleucine at the position equivalent to Leu47 in EGF. Ligand binding displacement studies on HER14 cells and 32D cells were performed as described previously (19, 25). Briefly, HER14 cells were grown to confluency and serial dilutions of unlabeled growth factors were added in the presence of 1 ng/ml of [^{125}I]-mEGF. After incubation for 2 h, cells were washed three times with phosphate-buffered saline (PBS) to remove unbound label, and incubated for 1 h in 1% Triton X-100 at room temperature prior to γ–counting. For D3, D13, and D23 cells, serial dilutions of growth factors were added in the presence of 1 ng/ml of [^{125}I]-NRG1β-opt and incubated for 2 h at 4ºC, after which unbound label was removed by centrifugation of the cells through a serum cushion. Cell bound radio-activity was measured

by γ–counting. In the case of [^{125}I]-WVR/EGF/IADIQ binding to D13 cells, radiolabeled ligand was added at 10 ng/ml.

Cell proliferation assay

For cell survival assays, D13, D23, and D24 cells were washed in RPMI-1640 medium to deprive them from IL-3. Subsequently, cells were seeded into 96-well tissue culture plates at a density of 5.0×10^4 cells/well in 0.1 ml of RPMI supplemented with 0.1% BSA, together with serial dilutions of recombinant growth factors. Cell survival was determined after 24 h of incubation at 37ºC using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described previously (28).

For mitogenic stimulation assays, HER14 and T47-14 cells were seeded into 24-wells plates at a density of 6.0×10^4 HER14 cells/well or 1.5×10^5 T47-14 cells/well, and grown for 24 h in serum-containing medium. The cells were serum-starved for 48 h, and subsequently serial dilutions of growth factor were added in 100 ml DMEM/BES. After 8 h (HER14) or 20 h (T47-14), 0.5 mCi of [^{3}H]-thymidine (TdR) was added in 50 ml of Ham's F12 medium and incorporation of [^{3}H]-TdR was determined 24 h after growth factor induction. Cells were then washed twice with PBS and incubated with methanol at room temperature. After 15 min the methanol was aspirated, and the dried cells were lysed in 1.0 ml of 0.2 N NaOH for 30 min at 37ºC as described (29). [^{3}H]-TdR incorporation was determined by liquid scintillation counting.

Western blotting

32D cells were serum-starved for 2 h prior to stimulation. Cells were exposed to growth factors for 3 h at 37 ºC and subsequently lysed in RIPA buffer containing freshly added protease inhibitors (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 1.5 mM EGTA, 1.5 mM $MgCl_2$, 1 mM PMSF, 5 mg/ml pepstatin A, 0.15 units/ml aprotinin, 5 mg/ml leupeptin, 2 mM Na_3VO_4). Lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 8% polyacrylamide gels, and proteins were transferred to 0.45 mm nitrocellulose membranes at 300 mA for 1 h. Nonspecific binding sites were blocked by incubation for 1 h with 5% nonfat dry milk in Tris/HCl-buffered saline/Tween (TBST; 10 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6). The membrane was then incubated overnight with a first antibody (1:2500 dilution in 5% nonfat dry milk in TBST or 5% bovine serum albumin in TBST) at 4 ºC, followed by a 1 h incubation with a peroxidase-conjugated secondary antibody (1:5000 dilution in 5% nonfat dry milk in TBST). After each incubation step, blots were washed three times in TBST with constant agitation. Blots were

developed using the enhanced chemi-luminescence (ECL) method. Standard molecular weight markers (range: 10-250 kDa; BioRad) were exposed to the same procedure. Antibodies used in this study are: anti-phosphorylated HER3/ErbB3 (Tyr1289) monoclonal antibody and anti-phosphorylated Akt (Ser473) polyclonal antibody, both from Cell Signaling Technology.

RESULTS

Generation of an EGF mutant with wild type affinity for ErbB1 and ErbB3 receptors

Previous phage display studies have shown that the binding affinity of EGF and of the EGF/TGFα chimera T1E for ErbB3 can be strongly enhanced by optimization of residues in both the N-terminal and C-terminal linear region. In the present study we have combined the optimized sequences of both linear regions from these phage display studies to construct recombinant EGF mutants with high affinity for both ErbB3 and ErbB1. Figure 1 shows the amino acid sequence alignment of the mutants used in this study. The generated mutants, which will be referred to as WVR/EGF/IFDWA and WVR/EGF/IADIQ, were produced as recombinant proteins in *E.coli* and tested for activity as fully purified, homogeneous proteins.

To analyze the effects of the introduced mutations on the affinity for ErbB1, a receptor binding competition study with [^{125}I]-mEGF was carried out on HER14 cells, a 3T3 cell line overexpressing human ErbB1. Figure 2A demonstrates that WVR/EGF/IADIQ shows similar ligand binding competition as wild type EGF. In contrast, WVR/EGF/IFDWA has only low binding affinity for ErbB1, even when compared with T1E. Most likely this results from the absence of a leucine or isoleucine at position 47 of the IFDWA-mutant, which is known to be essential for high affinity ErbB1 binding (13, 14, 30). No [^{125}I]-mEGF binding competition was observed with NRG1b or its affinity optimized mutant form NRG1β-opt (data not shown).

Figure 2B shows displacement curves for binding of [^{125}I]-NRG1β-opt to 32D cells expressing ErbB3 (D3 cells). The data show that WVR/EGF/IADIQ

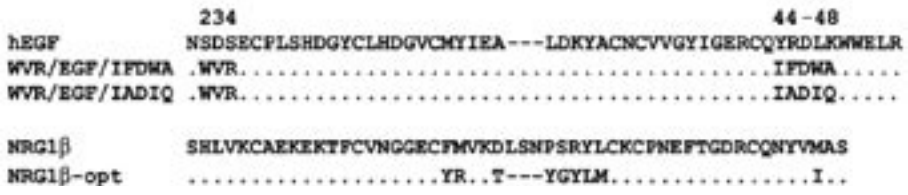

Figure 1. Alignment of amino acid sequences of hEGF, the EGF-like domain of human NRG1β, and the mutants used in this study.

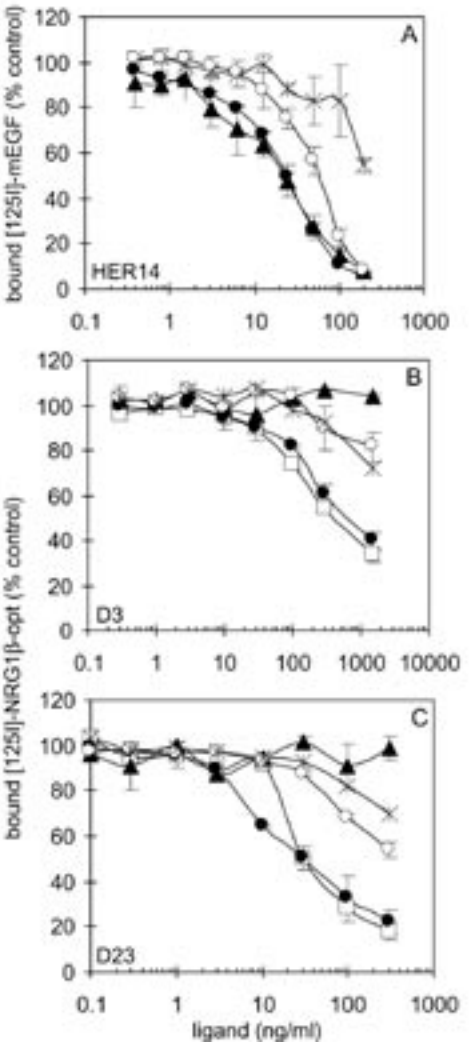

Figure 2. Ligand binding displacement analysis on cells expressing different ErbB receptors. (A) Displacement of [^{125}I]-mEGF binding to HER14 cells expressing human ErbB1; (B) Displacement of [^{125}I]-NRG1β-opt binding to 32D cells expressing ErbB3 (D3 cells); (C) Displacement of [^{125}I]-NRG1β-opt binding to 32D cells expressing ErbB2 and ErbB3 (D23 cells). Cells were incubated for 2 h at 4ºC with radiolabeled ligand in the presence of serial dilutions of unlabeled EGF (closed triangles), NRG1β (open squares), T1E (open circles), mutant WVR/EGF/IADIQ (closed circles), or mutant WVR/EGF/IFDWA (asterisks). Experiments were performed three times in duplicate, and are presented as mean ± sem.

competes for ErbB3 binding with near similar affinity as unlabeled NRG1β (half maximum concentration around 200 ng/ml), while EGF is fully inactive. T1E showed only low binding affinity, in agreement with previous data (19), while WVR/EGF/IFDWA behaved very similar to T1E. Unlabeled NRG1β-opt itself competed on these cells with a half maximum concentration of 10 ng/ml (not shown). In the case of 32D cells expressing both ErbB2 and ErbB3 (D23 cells), WVR/EGF/IADIQ and NRG1β showed again high binding affinity, while EGF and WVR/EGF/IFDWA showed no or only low binding affinity, as shown in Figure 2C. Thus, WVR/EGF/IADIQ has similarly high affinity as EGF for binding

to ErbB1, and as NRG1β for binding to ErbB3 and ErbB2-ErbB3 heterodimers. In this respect WVR/EGF/IADIQ therefore differs from T1E and biregulin which bind with high affinity to ErbB1 and ErbB2-ErbB3, but show only low binding affinity for ErbB3 alone. In contrast, the mutant WVR/EGF/IFDWA showed only low binding affinity to both ErbB1 and ErbB3, in spite of the fact that both the WVR sequence in the N-terminal and the IFDWA sequence in the C-terminal linear region gave rise to enhanced ErbB3 affinity in EGF mutants bound on phage. This indicates that sequences derived from affinity selected phage mutants cannot always be combined to produce recombinant EGF mutants with enhanced receptor affinity.

WVR/EGF/IADIQ efficiently activates both ErbB1 and ErbB3 receptors, but is unable to induce ErbB1-ErbB3 heterodimers

To test the ability of WVR/EGF/IADIQ to induce cell proliferation, mitogenic stimulation and cell survival assays were performed on cells carrying either ErbB1 or ErbB3 receptors. Figure 3A shows that WVR/EGF/IADIQ is equally potent as EGF in inducing [^{3}H]-thymidine incorporation into HER14, while NRG1β is fully inactive.

ErbB3 has an impaired kinase activity, and therefore its biological activity can only be assayed in cells containing a heterodimeric partner such as ErbB2. Figure 3B shows that WVR/EGF/IADIQ is even more potent than NRG1b in inducing cell survival of 32D cells containing both ErbB2 and ErbB3 (D23 cells), while EGF is fully inactive in this assay. In combination, these data show that the mutant WVR/EGF/IADIQ is at least as active as wild-type ligands in activating both ErbB1 and ErbB3 receptors, in agreement with the wild-type affinity of this mutant for both receptor members. In parallel with its low binding affinity for ErbB3, WVR/EGF/IFDWA appeared only a poor activator of ErbB2/ErbB3 heterodimers (data not shown).

Previous ErbB tyrosine phosphorylation assays have indicated that NRG1β is able to induce the formation of ErbB1/ErbB3 heterodimers in absence of an ErbB1 binding ligand (31). Since WVR/EGF/IADIQ can bind both receptors with high affinity, we examined if this mutant can induce the formation of ErbB1/ErbB3 heterodimers. Binding analysis on 32D cells carrying exclusively ErbB1 and ErbB3 (D13 cells), revealed that WVR/EGF/IADIQ fully competes with [^{125}I]-EGF for binding to ErbB1 (Figure 4A), and with [^{125}I]-NRG1β-opt for binding to ErbB3 (Figure 4B). This demonstrates that WVR/EGF/IADIQ can bind to both ErbB1 and ErbB3 on these cells. In order to show that this ligand can bind each of these receptors at the same time, [^{125}I]-WVR/EGF/IADIQ was added to D13 cells at a concentration of 10 ng/ml to occupy the majority of available receptors. Figure 4C shows that upon subsequent addition of unlabeled WVR/EGF/IADIQ full binding competition was obtained, compared to only 75-80% binding competition with saturating concentrations of EGF and 20-25% binding competition with

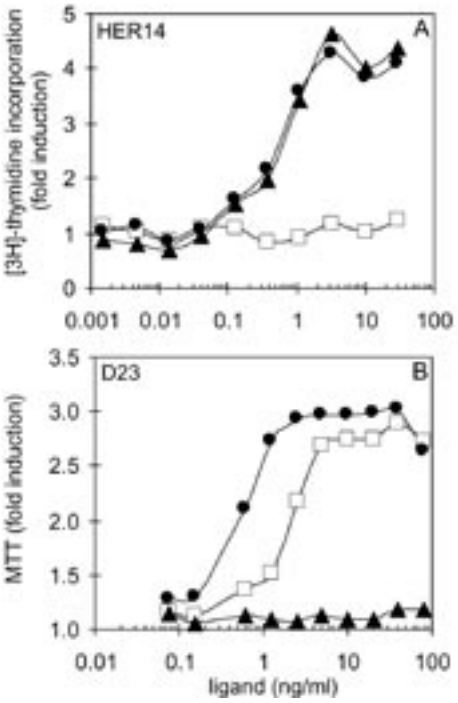

Figure 3. Survival and proliferation analysis on cells expressing different ErbB receptors. (A) Mitogenic stimulation of HER14 cells expressing human ErbB1 as monitored by incorporation of [^{3}H]-thymidine between 8 and 24 h after ligand addition; (B) Cell survival of 32D cells expressing both ErbB2 and ErbB3 (D23 cells) as measured in an MTT assay after 24 h. Cells were serum-starved before addition of serial dilutions of EGF (closed triangles), NRG1β (open squares), or the mutant WVR/EGF/IADIQ (closed circles). Experiments were performed twice in duplicate, and a representative experiment is shown.

saturating concentrations of NRG1β. This shows that WVR/EGF/IADIQ is able to bind both receptors on these cells, and is selectively removed from ErbB1 by EGF and from ErbB3 by NRG1β. Quantitatively, these data agree with the known four-fold excess of ErbB1 over ErbB3 receptors in the cell line.

Figure 5A shows in an MTT assay that NRG1β is a potent inducer of cell survival in D13 cells, as a result of the formation of ErbB1/ErbB3 heterodimers in which only ErbB3 is ligand-occupied. EGF itself is much less potent in the this assay than NRG1β, particularly at concentrations above 10 ng/ml, which indicates that in these cells NRG-induced ErbB1/ErbB3 heterodimers generate more potent survival signals than EGF-induced ErbB1 homodimers. Figure 5A also shows that WVR/EGF/IADIQ is unable to induce the potent cell survival levels of NRG1β in D13 cells, but instead mimics the behavior of EGF. This indicates that WVR/EGF/IADIQ may bind both ErbB1 and ErbB3 in these cells, but is unable to form functional ErbB1/ErbB3 heterodimers. The low affinity mutant WVR/EGF/IFDWA induced similar effects as EGF and WVR/EGF/IADIQ, but only when added at elevated concentrations (data not shown).

To examine if ligand binding to ErbB1 interferes with the NRG1β-induced ErbB1/ErbB3 heterodimer formation, we administered NRG1β in combination with either EGF or WVR/EGF/IADIQ to D13 cells. Figure 5B shows that WVR/EGF/IADIQ behaves similar to EGF and is able to impair the NRG1β-induced high cell survival in D13 cells in a dose-dependent manner, with a

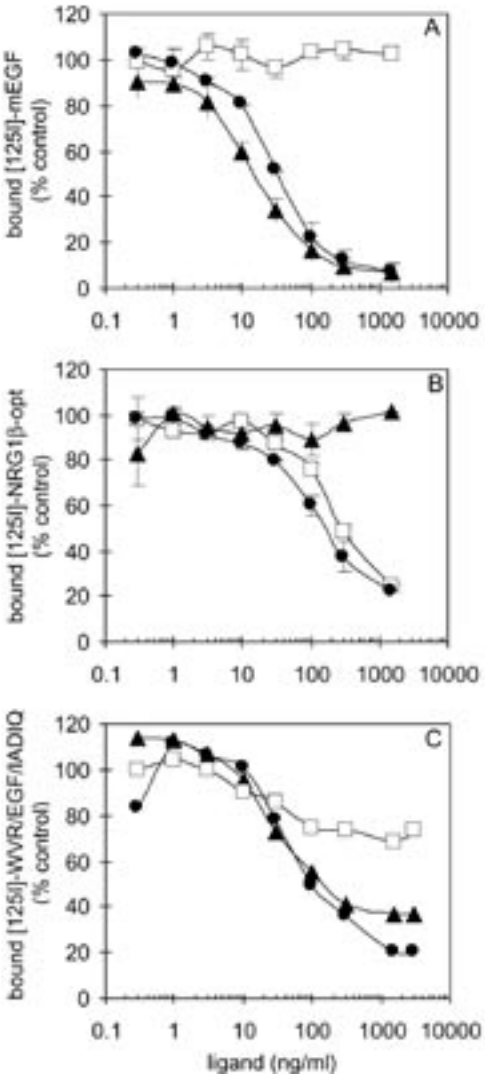

Figure 4. Ligand displacement analysis on 32D cells expressing both ErbB1 and ErbB3 (D13 cells). (A) Displacement of [^{125}I]-mEGF binding by unlabeled EGF (closed triangles), NRG1β (open squares), or the mutant WVR/EGF/IADIQ (closed circles). Cells were incubated for 2h at 4ºC with 1 ng/ml radiolabeled ligand in the presence of serial dilutions of the above unlabeled growth factors. Unbound ligand was removed by sedimentation of the cells through a serum cushion, after which cell-bound radioactivity was determined. (B) Displacement of 1 ng/ml of [^{125}I]-NRG1β-opt binding by unlabeled EGF, NRG1β or WVR/EGF/IADIQ (symbols as above). (C) Displacement of [^{125}I]-WVR/EGF/IADIQ binding by unlabeled EGF, NRG1β or WVR/EGF/IADIQ (symbols as above). Cells were incubated with 10 ng/ml of [^{125}I]-WVR/EGF/IADIQ to saturate available ErbB1 and ErbB3 receptors. Experiments were performed three times in duplicate, and are presented as mean ± sem.

half maximum effect at 1-2 ng/ml. Pretreatment of D13 cells with NRG1β for 1 h did not affect the inhibitory effect of EGF or WVR/EGF/IADIQ (data not shown). So, in spite of the fact that WVR/EGF/IADIQ can bind both ErbB1 and ErbB3, it is unable to mimic and even blocks the cell survival signals induced by NRG1β in D13 cells. Most likely both EGF and WVR/EGF/IADIQ induce the formation of ErbB1 homodimers in these cells and thereby inhibit the formation of NRG1β-induced ErbB1/ErbB3 heterodimers.

Figure 6 (upper panel) shows that NRG1β is able to induce tyrosine phosphorylation of ErbB3 in D13 cells, most likely as a result of activation of

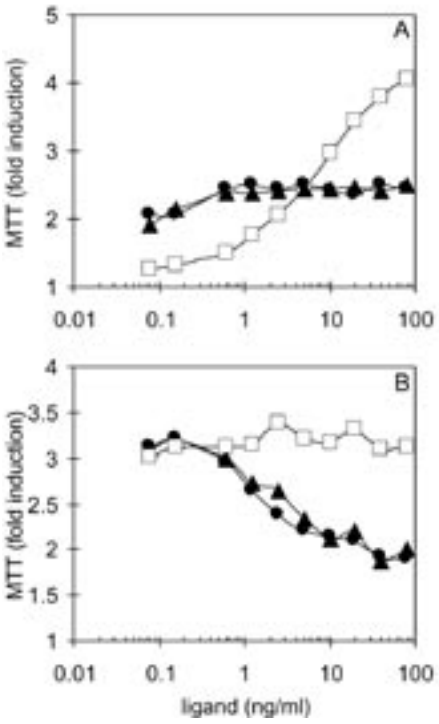

Figure 5. Growth factor- induced survival of 32D cells expressing both ErbB1 and ErbB3 (D13 cells). Growth factors were added to serum-starved D13 cells, and cell survival was measured after 24 h in an MMT assay. (A) Addition of serial dilutions of EGF (closed triangles), NRG1β (open squares), or the mutant WVR/EGF/IADIQ (closed circles). (B) Combined addition of 50 ng/ml of NRG1β with serial dilutions of EGF (closed triangles), the mutant WVR/EGF/IADIQ (closed circles), or without additional ligand (open squares). Experiments were performed twice in duplicate, and a representative experiment is shown.

the ErbB1 tyrosine kinase in the ErbB1/ErbB3 complex. Upon addition of EGF, the NRG1β-induced phosphorylation of ErbB3 decreased in a dose-dependent manner, whereas the downstream Akt phosphorylation remained unchanged. This indicates that EGF is indeed able to dissociate ErbB1 receptors from existing ErbB1/ErbB3 heterodimers, in favor of the formation of ErbB1 homodimers. Figure 6 (lower panel) shows that WVR/EGF/IADIQ on its own is unable to induce ErbB3 tyrosine phosphorylation in these cells. So although WVR/EGF/IADIQ can bind to both ErbB1 and ErbB3 independently, it is unable to form a functional ErbB1/ErbB3 heterodimeric complex. This figure also shows that WVR/EGF/IADIQ mimics EGF in its ability to block NRG1β-induced phosphorylation of ErbB3 in a dose-dependent manner, which indicates that in D13 cells WVR/EGF/IADIQ binds both ErbB1 and ErbB3, but only signals through ErbB1 homodimers. It thus appears that active ErbB1/ErbB3 heterodimers are exclusively formed when ErbB3 is present in its liganded form and ErbB1 as coreceptor in an unliganded conformation.

WVR/EGF/IADIQ can induce proliferation via ErbB4

Since the requirements in NRG1 for ErbB3 and ErbB4 binding are largely overlapping, we next analyzed the binding and activation characteristics of WVR/EGF/IADIQ towards cells expressing ErbB4. In ligand binding displacement experiments on T47-14 cells, a 3T3 cell line overexpressing human ErbB4, and D24 cells, which contain both ErbB4 and ErbB2, we did not detect any displacement of [^{125}I]-NRG1β-opt binding by WVR/EGF/IADIQ up to concentrations of 500 ng/ml (data not shown). In spite of this very low binding affinity for ErbB4, we observed that WVR/EGF/IADIQ induced similar mitogenic activity in T47-14 cells as NRG1β, while EGF was inactive (Figure 7A). Moreover, in D24 cells WVR/EGF/IADIQ induced similar cell survival levels as NRG1β, while EGF was only weakly active (Figure 7B). So, in spite of its low binding affinity WVR/EGF/IADIQ is able to activate signal transduction pathways through ErbB4. In combination with the above data it thus appears that WVR/EGF/IADIQ behaves similar to NRG1β and unlike EGF in ErbB4 containing cells (D24 and T47-14), whereas in D13 cells it behaves similar to EGF and unlike NRG1β.

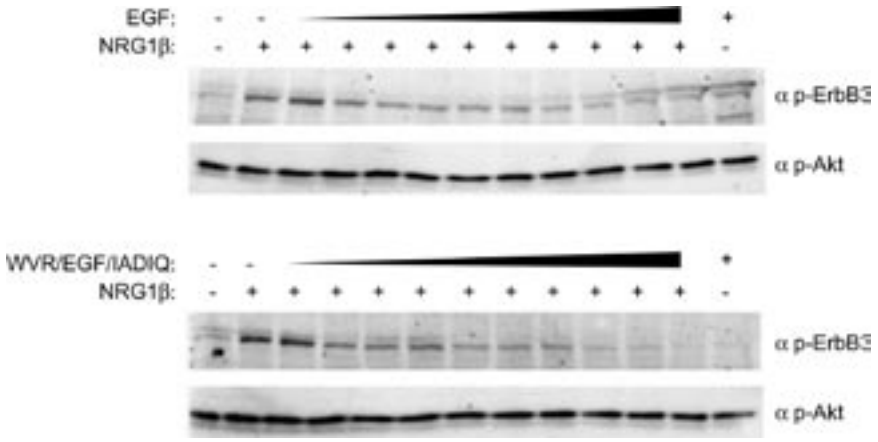

Figure 6. Growth factor-induced inhibition of NRG1β-induced ErbB3 activation in cells expressing ErbB1 and ErbB3 (D13 cells). Cells were stimulated for 3 h without (-) or with 50 ng/ml (+) of either NRG1β, EGF or WVR/EGF/IADIQ; in addition cells were treated with a combination of 50 ng/ml of NRG1β (+) and increasing concentrations (0.1-0.25-0.5-0.75-1.0-2.5-5-10-25-50 ng/ml) of EGF (upper lane) or WVR/EGF/IADIQ (lower lane). Immunoblotting of whole cell lysates was performed with an anti-p-ErbB3 antibody or an anti-p-Akt antibody.

DISCUSSION

In this study we show that the N- and C-terminal linear regions of EGF-like growth factors play an important role in their receptor binding specificity. An EGF mutant containing N-terminal and C-terminal sequences optimized for ErbB3 binding, designated WVR/EGF/IADIQ, gained wild-type affinity for cells expressing ErbB3, while it maintained wild-type affinity for ErbB1. Similar to the natural ligands, this dual-specific mutant is fully capable to activate ErbB1 homodimers and ErbB2/ErbB3 heterodimers, while it is also active on cells expressing ErbB4. In cells expressing both ErbB1 and ErbB3, WVR/EGF/IADIQ could compete with both EGF and NRG1β for receptor binding, but induced only EGF-like survival activity, most likely because it prefers the formation of ErbB1 homodimers over ErbB1/ErbB3 heterodimers. Apparently the dual-specific binding property of this mutant does not contribute to the formation of functional ErbB1/ErbB3 heterodimers. These data furthermore show that high affinity binding to both ErbB1 and ErbB3 is not mutually exclusive, in spite of the fact that no naturally occurring ligand exists with this dual specificity.

From site-directed mutagenesis studies on EGF and TGFα, much information is known about residues that are essential for high affinity binding to ErbB1 (12-14). In the linear C-terminal region particularly the leucine at position 47 is of central importance. For binding to ErbB3, we observed in

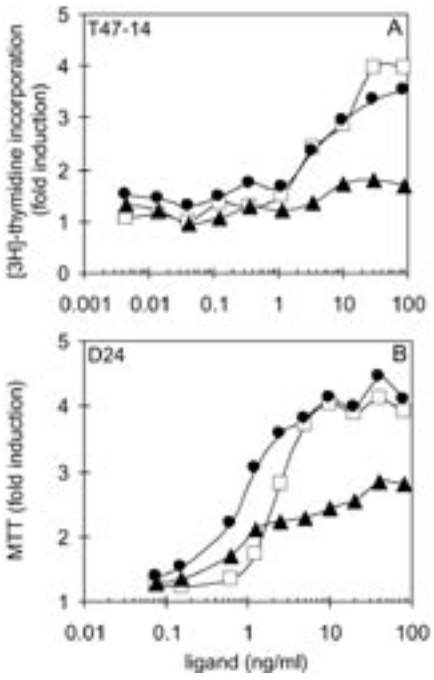

Figure 7. Survival and proliferation analysis on cells expressing only ErbB4 (T47-14) or 32D cells expressing both ErbB4 and ErbB2 (D24). (A) Mitogenic stimulation of T47-14 cells as monitored by incorporation of [^{3}H]-thymidine between 8 and 24 h after ligand addition; (B) Cell survival of D24 cells as measured in an MTT assay 24 h after ligand addition. Cells were serum-starved before addition of serial dilutions of EGF (closed triangles), NRG1β (open squares), or the mutant WVR/EGF/IADIQ (closed circles). Experiments were performed twice in duplicate, and a representative experiment is shown.

our phage display study a preference for isoleucine or tryptophan at the equivalent position (*22*). The observation that WVR/EGF/IADIQ retained wild-type affinity for ErbB1 whereas WVR/EGF/IFDWA showed strongly decreased affinity, results therefore most likely from the fact that at position 47 a tryptophan is much less well tolerated than an isoleucine for high affinity ErbB1 binding. The observation that WVR/EGF/IFDWA is only a low affinity ligand for ErbB3 is puzzling, since the N-terminal sequence WVR and the C-terminal sequence IFDWA have been selected individually in phage display studies as requirements for high binding affinity to ErbB3.

Intriguingly, in spite of its high affinity to both ErbB1 and ErbB3, WVR/EGF/IADIQ is unable to activate ErbB1/ErbB3 heterodimers. ErbB3 differs from other ErbB members in that it has an impaired tyrosine kinase, and as a result it depends for activity on heterodimerization with another ErbB member. The orphan ErbB2 is the preferred coreceptor for ErbB3, and ErbB2 heterodimer formation strongly enhances the binding affinity of ligands to ErbB3. As a consequence, even low affinity ErbB3 ligands such as NRG1α, T1E and biregulin, are potent activators of ErbB2/ErbB3 heterodimers. However, only high affinity ErbB3 ligands, such as NRG1β, are able to recruit ErbB1 as dimerization partner and to activate cells expressing both ErbB3 and ErbB1, even in absence of ErbB2 (31). Since NRG1β has no detectable affinity for ErbB1, this coreceptor must be present in an unliganded form in the ErbB1/ErbB3 complex. It remains unclear how ErbB1 is recruited into this complex. Crystallographic studies have shown that in the absence of ligand, ErbB receptors are in a closed conformation and thereby unable to dimerize. However, it has been suggested that a fraction of unliganded ErbB1 receptors may already exist in an open although inactive conformation (32). It could be speculated that upon NRG1β binding ErbB3 receptors undergo a change from a closed to an open conformation, and subsequently are able to heterodimerize with available unliganded ErbB1 receptors in the open conformation. Formation of such a ErbB1/ErbB3 heterodimeric complex could subsequently stabilize the open conformation of ErbB1 and activate its tyrosine kinase. Although theoretically possible, ErbB1/ErbB3 heterodimeric complexes are not observed when ErbB1 and ErbB3 are both liganded, either by treatment with WVR/EGF/IADIQ or in the presence of both NRG1β and EGF. Pinkas-Kramarski and coworkers found that EGF is able to impair the NRG1β-induced survival in D13 cells when added simultaneously, probably through a preference for ErbB1 homodimers (23). Here, we show that even existing ErbB1/ErbB3 heterodimeric complexes formed in the presence of NRG1β are dissociated upon addition of EGF or WVR/EGF/IADIQ, although this latter ligand shows wild-type affinity for both ErbB1 and ErbB3. It will be interesting to determine if the amount of ErbB1/ErbB3 heterodimeric complexes induced by NRG1β is indeed limited by the fraction of ErbB1 receptors that, in the unliganded state, is present in an open conformation.

The ligand binding displacement curves in Figure 4A indicate that half-maximum saturation of ErbB1 receptors in D13 cells requires 10-20 ng/ml of

EGF. In contrast, these concentrations of EGF are more than sufficient to fully withdraw ErbB1 receptors from NRG1β-induced ErbB1/ErbB3 heterodimers, as shown in Figure 5B. This suggests that in the presence of 10-20 ng/ml of EGF, unliganded ErbB1 receptors are still present that are, however, unable to form heterodimers with NRG1β-bound ErbB3 receptors. One explanation could be that the unliganded ErbB1 receptors in the open conformation have a higher EGF binding affinity than the bulk of ErbB1 receptors in the closed conformation, and that consequently relatively low concentrations of EGF are enough to bind this subset of receptors and induce them to form ErbB1 homodimers. Alternatively, ErbB1 receptors in the open conformation could obtain their enhanced binding affinity due to the stabilization of their conformation in the ErbB1/ErbB3 complex. Finally, there is increasing evidence that ErbB receptors can be distributed in a heterogeneous manner in the plasma membrane, e.g. by accumulation in lipid rafts (33). It can therefore also not be excluded that ErbB3 receptors are present in membrane microdomains that also contain ErbB1 receptors with enhanced binding affinity.

Crystallographic data in combination with site-directed mutagenesis studies have indicated that subdomain III of ErbB1 is essential for high affinity binding of EGF, while in the case of ErbB3 subdomain I is of primary importance for NRG1β binding (12-18). This suggests that ErbB1 and ErbB3 bind their ligands in a different manner. Crystal structures have been published of EGF- and TGFα-liganded ErbB1 receptors, but so far only the conformation of unliganded ErbB3 is known. Because of its high binding affinity to both ErbB1 and ErbB3, WVR/EGF/IADIQ would be an ideal candidate to compare the ligand binding characteristics of ErbB1 and ErbB3 by studying their crystal structures bound to the same ligand.

ACKNOWLEDGMENTS

We thank M. H. Kraus (University of Alabama at Birmingham, Birmingham, USA) and Y. Yarden (Weizmann Institute of Science, Rohovot, Israel) for the kind gift of the T47-14 and 32D cells, respectively. Pascal Hommelberg is acknowledged for excellent technical assistance in making the NRG1β-opt construct.

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5

Role of the C-terminal linear region of EGF-like growth factors in ErbB selectivity

Growth Factors, 27, 163-172 (2009)

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ABSTRACT

EGF-like growth factors bind their ErbB receptors in a highly selective manner. Recently we have shown that the sequence YYDLL in the C-terminal linear region is compatible with binding to all ligand-binding ErbB receptors. In the present study we show that introduction of the YYDLL sequence into the ErbB1 specific ligands EGF and TGFα broadened their receptor specificity towards ErbB4. Upon introduction of the YYDLL sequence into EREG, which by itself binds ErbB1 and ErbB4 but not ErbB3, its binding specificity was broadened to ErbB3, concomitant with enhanced affinity for ErbB4. Introduction of the YYDLL sequence into NRG1β resulted in a ten-fold increase in affinity for ErbB3, without affecting its receptor specificity. Remarkably, the strongly enhanced affinity for ErbB3 negatively influenced their mitogenic activity towards cells coexpressing ErbB2 and ErbB3. These observations are discussed in terms of the optimized ErbB affinity, selectivity and mitogenic potential that have taken place during evolution.

INTRODUCTION

The epidermal growth factor (EGF) family of polypeptide growth factors and their corresponding ErbB receptors show an interesting pattern of evolutionary diversification. The eleven growth factor and four receptor genes observed in mammals have evolved from a single receptor-ligand signaling complex, that is still present in invertebrates such as C. elegans. Studies on the structure-function relationship of EGF-like growth factors have indicated that during evolution optimisation of receptor binding specificity has not coincided with optimisation of receptor binding affinity (1-3). As a consequence, the molecular basis for receptor binding affinity and specificity of EGF-like growth factors is still poorly understood.

The ErbB family of receptor tyrosine kinases consists of ErbB1, ErbB2, ErbB3 and ErbB4, which all play a crucial role in the growth control of mammalian cells and the development of multicellular organisms (4). Depending on their ErbB binding specificity, EGF-like growth factors can be divided into three subfamilies: EGF, transforming growth factor-α (TGFα), amphiregulin (AREG) and epigen (EPGN) form a subfamily that binds exclusively to ErbB1, while the subfamily consisting of betacellulin (BTC), epiregulin (EREG) and heparin-binding EGF (HB-EGF) binds not only ErbB1, but also ErbB4. Finally the neuregulins form a subfamily of which NRG1 and NRG2 bind both ErbB3 and ErbB4, while NRG3 and NRG4 appear specific for ErbB4 (5). ErbB2 does not bind ligand by itself, but is the preferred heterodimerization partner for all liganded ErbBs and is particularly important for ErbB3 signaling which has a defective tyrosine kinase domain (6).

Phylogenetic tree analysis of vertebrate ErbB genes suggests that

during evolution an initial whole genome duplication has resulted in the generation of two receptor types, i.e. an ErbB1/ErbB2 ancestor and an ErbB3/ErbB4 ancestor (Stein and Staros 2000). A subsequent whole genome duplication has facilitated the formation of the current four distinct receptor genes. Based on similar analysis it has also been proposed that TGFα and BTC have arisen from a common ancestor gene, similarly as AREG and HB-EGF, as well as EPGN and EREG (7). Remarkably in all these three cases the ancestral gene has evolved into a gene encoding an ErbB1–specific ligand and a gene encoding a ligand that binds both ErbB1 and ErbB4. Apparently, ligands and receptors have coevolved under evolutionary pressure to maintain multiple sets of ligands with similar ErbB binding diversity (1).

EGF-like growth factors consist of approximately 50 amino acids and share a conserved spacing of six cysteines that form three disulphide bridges, thereby giving rise to an N-terminal linear region, an A-loop, B-loop and C-loop, and a C-terminal linear region (8-9). From crystal structure analysis of the EGF/ErbB1 and the TGFα/ErbB1 complex, it is known that EGF-like growth factors contain approximately 20 residues that directly interact with their receptor (10-11). These residues, which we have referred to as positive constraints, form the basis for the observed high affinity receptor interaction. However, in a previous study we have provided evidence that EGF-like growth factors contain in addition a limited number of residues that are not essential for high affinity binding to their cognate receptor, but that prevent the unintended binding to other ErbB receptors (1). These residues, which we have referred to as negative constraints, appear to be conserved in evolution, and therefore we have hypothesized that negative constraints form the basis for the observed receptor specificity of EGF-like growth factors (1).

In previous studies we have identified three such negative constraints in EGF and TGFα, which prevent them from binding to ErbB3 and ErbB4. Using domain-exchange strategies, we have shown that mutation of Ser2/Asp3 in the N-terminal linear region of EGF into the corresponding residues His2/Phe3 from TGFα is sufficient to give EGF considerable affinity for ErbB3 and ErbB4 (2). Similarly the point mutation E26L in the B-loop of TGFα has been shown to broaden its affinity to at least ErbB3 (12). Based on a phage display affinity optimisation of the C-terminal linear region, we have recently shown that Arg45 in EGF facilitates binding to ErbB1, but acts as a negative constraint for binding to ErbB3 and ErbB4 (1). Moreover, we could show that the sequence YYDLL in the C-terminal linear region optimizes positive constraints and releases negative constraints for ligand binding to ErbB1, ErbB3 and ErbB4. Upon elimination of all known negative constraints from EGF, we subsequently designed a mutant designated T1E/YYDLL, which had lost all receptor binding selectivity and displayed high affinity interaction with all ligand binding ErbB receptors. This designed ligand, also referred to as panerbin, appeared very useful for determination of the density of the various ErbB receptors on tumor cells in a single radio-receptor assay (1).

In the present study we have investigated the role of the C-terminal

linear region on ErbB binding affinity and specificity in more detail. We therefore introduced the optimised sequence YYDLL into an number of naturally occurring EGF-like growth factors and studied the effects on ErbB affinity and specificity. Our results show that introduction of this sequence into NRG1β strongly enhanced its affinity for ErbB3, without affecting receptor specificity. In the case of the ErbB1-specific ligands EGF and TGFα, it broadened receptor specificity towards ErbB4, without affecting their ErbB1 binding affinity. A combination of the two effects was observed for EREG, which by itself binds ErbB1 and ErbB4 with intermediate affinity, but lacks affinity for ErbB3. Upon introduction of the YYDLL sequence, EREG broadened its binding specificity towards ErbB3 and enhanced its affinity for ErbB4. Using homology modeling of receptor-ligand complexes we could show that EREG with its aberrant conformation (13) contains less positive constraints for ErbB binding, which in the case of ErbB3 and ErbB4 is enhanced upon introduction of the YYDLL sequence. These observations are discussed in terms of the molecular mechanisms that have resulted in optimization of ErbB binding affinity, selectivity and mitogenic potential, as has been maintained during evolution.

EXPERIMENTAL

Cell lines

Interleukin 3-dependent murine 32D hematopoietic progenitor cells transfected with distinct human ErbB-encoding plasmids were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and 0.25 ng/ml interleukin-3 (Promega, Madison, WI) and subsequently kept under continuous selection using 0.6 mg/ml G418 (Calbiochem). In this study we used D3 cells (32D cells transfected with the human ErbB3 gene), D23 cells (32D cells transfected with both the human ErbB2 and ErbB3 gene) and D24 cells (32D cells transfected with both the human ErbB2 and ErbB4 gene). HER14 cells (mouse fibroblasts overexpressing human ErbB1) and T47-14 cells (mouse fibroblasts overexpressing human ErbB4) were cultured in gelatinized flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS).

MTT assay

32D cells were deprived of interleukin-3 by washing and subsequently resuspended in serum-free RPMI 1640 medium supplemented with 0.1% bovine serum albumin (BSA). Cells were seeded in 96-wells plates at a density of $5.0x10^4$ cells/well in a total volume of 0.1 ml, in the presence or absence of serial dilutions of recombinant growth factors. Cell survival was

determined after 24 hours of incubation at 37°C using the colorimetric 3-(4,5-dimethylthiazzol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Two hours after the addition of 50 mg/well of MTT, cells were lysed in 0.1 ml acidic isopropanol and carefully resuspended. Plates were read at 550-655 nm, and duplicate data points were plotted as fold induction over untreated control wells.

Mitogenic Assays

DNA replication of T47-14 cells upon ligand stimulation was monitored by [^{3}H]-thymidine incorporation. Cells were seeded in 24-wells plates at a density of 3.0×10^4 cells/well in 1 ml of DMEM supplemented with 10% NCS. After 24 hours, the medium was replaced by 1 ml of DF medium (1:1 mixture of DMEM and Ham's F12 medium), supplemented with 30 nM Na_2SeO_3, 10 µg/ml human transferrin, and 0.5% BSA. Cells were thus serum-starved for an additional 24 hours, after which serial dilutions of growth factors were added in 50 µl of BES-buffered DMEM/0.1%BSA (pH 6.8), as described (van der Burg and others 1988). Eight hours later, 0.5 µCi of [^{3}H]-thymidine was added in 50 µl of Ham's F12 medium, and incorporated thymidine was determined 24 hours after growth factor addition. Cells were incubated for 15 min with methanol to remove non-incorporated cytoplasmic [^{3}H]-thymidine and subsequently lysed by addition of 1 ml of 2 N NaOH for 1 hour at 37°C. Radioactivity was determined by liquid scintillation counting.

Expression and Purification of Mutant Growth Factors

Recombinant mutant growth factors were expressed as protein A-tagged fusion proteins in the protease K-deficient Escherichia coli strain KS474 and isolated from the periplasmic space as described (14). Growth factors were purified by means of affinity chromatography using IgG-Sepharose, followed by Factor-X cleavage, an additional round of affinity chromatography to remove the protein A-tag and a final reverse phase (RP)-HPLC purification step to remove disulphide bridge mismatches. The amount of growth factor was calculated from the peak area (absorption at 229 nm) in the RP-HPLC chromatogram, using natural mEGF as a standard. The affinity for ErbB1 was measured in an [^{125}I]-mEGF binding competition assay on HER14 cells. Affinity for ErbB3 or ErbB4 was determined in an [^{125}I]-NRG1β binding competition assay on D3, D23 and D24 cells, respectively.

Ligand Binding Displacement Experiments

Recombinant EGF and NRG1β were radiolabeled enzymatically to a specific activity of 1.1 Ci/μmol (15). Ligand binding displacement analyses were performed either using a suspension of 5.0×10^6 32D cells or confluent monolayers of HER14 or T47-14 cells. 32D cells were washed once with RPMI supplemented with 0.5% BSA and subsequently incubated for 2 hours at 4 ^{o}C with serial dilutions of unlabeled ligand in the presence of 0.17 nM [^{125}I]-NRG1β. Cells were then washed once with binding buffer and loaded onto a serum cushion to remove the unbound label. Subsequently, cells were quickly spun down at 2000 rpm, and cell surface-bound radioactivity was determined by γ-counting. HER14 or T47-14 cells were grown to confluency, and serial dilutions of unlabeled growth factors were added in the presence of 0.17 nM of [^{125}I]-mEGF or 0.17 nM [^{125}I]-NRG1β, respectively. After incubation for 2 hours at 4 ^{o}C, the cells were washed three times with phosphate-buffered saline (PBS) in order to remove the unbound label and subsequently incubated for 1 hour in 1% Triton X-100 at room temperature prior to γ-counting.

Homology modeling of EREG in complex with ErbB receptors

Homology models for ErbB-ligand complexes were based on the published crystal structures of the ErbB1-EGF and ErbB1-TGFα (10-11) complex as a template. Validation of the models with WHAT CHECK (Hooft and others 1996) identified template 1MOX, obtained from the ErbB1-TGFα complex, as the best choice of both receptor and ligand. The amino acid side chains in the final models were positioned using SCWRL (16). Subsequently, the models were refined in YASARE using Yamber2 force field and associated protocol (17) until the WHAT IF (18) quality indicators (Ramachandran plot, backbone conformation, and three-dimensional packing quality) converged. For analysis of EREG in complex with ErbB receptors, use was made of the energy-minimized average NMR structure of EREG (PDB 1K37). For ErbB complexes with NRG1β and EREG/YYDLL the TGFα crystal structure 1MOX was used. Coordinate files of the final models are available from the authors upon request.

RESULTS

Exchange of the C-terminal linear region of EGF-like growth factors by YYDLL affects their ErbB affinity and specificity

In order to investigate the effect of the C-terminal linear region on the ErbB specificity and affinity of EGF-like growth factors, we introduced the YYDLL sequence into EGF, TGFα, NRG1β, EREG and the EGF/TGFα chimera T1E, whereby the existing Glu or Gln residue following the sixth cysteine was maintained. T1E is an artificial ligand that consists of TGFα sequences N-terminal and EGF sequences C-terminal of the first cysteine (19). T1E/YYDLL has previously been characterised by us as a ligand that binds ErbB1, ErbB3 and ErbB4 with high affinity (1) .

Figure 1A shows the ability of the wild-type ligands to compete with radiolabeled EGF for binding to the ErbB1 overexpressing cell line HER14. EGF, TGFα and T1E are high affinity ligands for ErbB1, while EREG showed intermediate and NRG1β no affinity for this receptor (see Table I for IC50 values). Introduction of the YYDLL sequence (Figure 1B) hardly affected the binding affinity of these ligands for ErbB1 (Table I), indicating that the wild-type sequence of the C-terminal linear region of EGF and TGFα is already optimal for ErbB1 binding, while the affinity of EREG and NRG1β for ErbB1 appears to be limited by residues outside the C-terminal linear region.

Figures 1C and 1D show the relative binding affinity of this set of ligands for ErbB3, as measured in a binding competition assay with radiolabeled NRG1β to 32D cells expressing only ErbB3 (D3 cells). Of the wild type ligands only NRG1β showed clear binding affinity, while upon introduction of the YYDLL sequence a considerable gain in affinity was observed for EREG, T1E and NRG1β itself (Table I). On the other hand, EGF and TGFα did not show any affinity for ErbB3, also not after introduction of the YYDLL sequence. Thus, both the unstructured N-terminal linear region of EGF and the presence of Glu26 in TGFα appears sufficient to prevent the unintended binding to ErbB3. The observation that introduction of YYDLL into NRG1β resulted in a more than ten-fold increase in affinity for ErbB3, leading to an extremely low IC50 of 0.06 nM, confirms previous studies that the affinity of NRG1β for its native receptor ErbB3 can be strongly increased by optimizing its C-terminal sequences (2). It remains unclear, however, why not a similar increase in affinity is observed for binding to ErbB2/ErbB3 heterodimers. In addition our data show that also the ErbB1 and ErbB4 specific ligand EREG gains affinity for ErbB3 upon introduction of the YYDLL sequence in the C-terminal linear region.

Figure 1E shows the ability of the various ligands to compete with radiolabeled NRG1β for binding to the well-characterized ErbB4 overexpressing cell line T47-14. With the exception of TGFα, the wild-type ligands showed detectable affinity for ErbB4, in the following order: NRG1β > T1E > EREG > EGF. Introduction of an YYDLL tail into TGFα (Figure 1F) also allowed binding

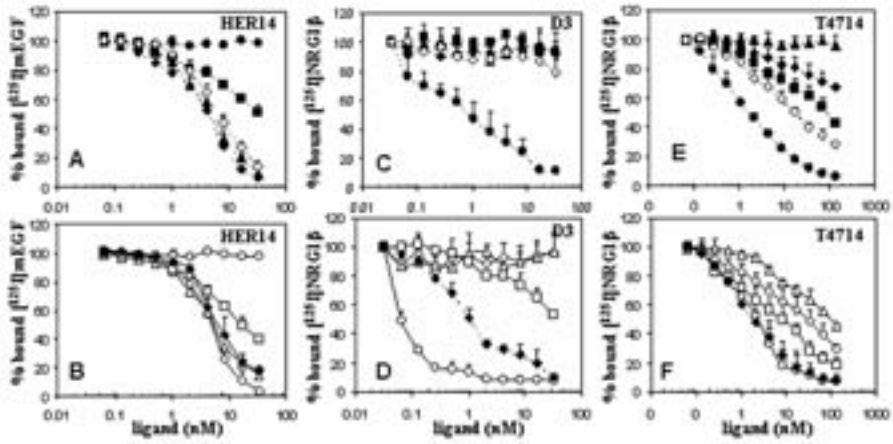

Figure 1 Binding affinity of wild-type EGF-like growth factors and YYDLL mutants for ErbB1, ErbB3 and ErbB4
(**A,B**) Ligand binding displacement analysis on ErbB1 over-expressing HER14 cells treated with 0.17 nM [^{125}I]-EGF. (**C,D**) Ligand binding displacement analysis on ErbB3 overexpressing D3 cells treated with 0.17 nM [^{125}I]-NRG1β. (**E,F**) Ligand binding displacement analysis on ErbB4 overexpressing T47-14 cells treated with 0.17 nM [^{125}I]-NRG1β. Cells were incubated with increasing amounts of the ligands EGF (···◆···), EGF/YYDLL (−◇−), TGFα (···▲···), TGFα /YYDLL (−△−), EREG (···■···), EREG/YYDLL (−□−), NRG1β (···●···), NRG/YYDLL (−○−), T1E (···◇···) and T1E/YYDLL (−◆−). Values of bound radiolabeled ligand without competition of unlabeled ligand was set on 100%. Results are the mean of at least two independent experiments performed in duplicate.

of this ligand to ErbB4, making this the first TGFα mutant that binds ErbB4. Both EGF and EREG changed from a low to a high affinity ligand for ErbB4 upon introduction of the YYDLL sequence (IC50 < 35 nM; Table I). Introduction of this sequence into T1E enhanced its affinity for ErbB4 to the level of NRG1β. In contrast to the observation on ErbB3, the affinity of NRG1β for ErbB4 could not be further enhanced by introduction of the YYDLL sequence. These data combined show that introduction of an YYDLL C-terminal tail particularly affects the ability of ligands to bind ErbB3 and ErbB4. More specific, NRG1β gained affinity without altering its receptor specificity, while EREG broadened its receptor specificity towards ErbB3, and EGF and TGFα towards ErbB4.

ErbB2 controls the affinity and mitogenic potential of ErbB3 binding ligands

We have previously shown that the mitogenic potential of ErbB1 ligands is a complex function of receptor affinity, receptor density and ligand depletion (20). In the case of ErbB3, mitogenic activation requires heterodimer formation with ErbB2. We therefore first studied the effect of

introduction of the YYDLL sequence on the binding affinity of NRG1β and T1E towards cells containing both ErbB2 and ErbB3 (D23 cells), and subsequently measured their mitogenic potential towards these cells. Figure 2A shows that wild-type NRG1β and NRG/YYDLL bind with equally high affinity to D23 cells (see Table I for IC50 values). This implies that introduction of the YYDLL sequence into NRG1β does not result in enhanced ErbB3 affinity, following the formation of ErbB2/ErbB3 heterodimers. The IC50 of both NRG1β and NRG/YYDLL on D23 cells is around 0.5 nM, which is 10-fold higher than that of NRG/YYDLL on D3 cells (Figures 1C and 1D). This is remarkable since according to current models heterodimer formation with ErbB2 will stabilize ErbB3-ligand interaction, resulting in enhanced binding affinity.

In contrast, the IC50 of T1E for binding to ErbB2/ErbB3 heterodimers is reduced 10-fold upon introduction of the YYDLL sequence, indicating that the affinity of T1E for ErbB2/ErbB3 heterodimers is suboptimal. Figure 2B shows that the mitogenic activity of NRG1β (EC50 50 pM) is reduced upon introduction of YYDLL (EC50 250 pM), while that of T1E (EC50 250 pM) is enhanced upon introduction of YYDLL (EC50 33 pM). These data indicate that the mitogenic activity of ligands for ErbB2/ErbB3 heterodimers is not a direct reflection of the their receptor binding affinity. Since the ErbB2-ErbB3 is the only possible signaling dimer on D23 cells, these results indicate that NRG/YYDLL is less efficient than NRG1β in inducing ErbB2-ErbB3 heterodimers.

	IC50 (nM)					EC50 (pM)		
	ErbB1	ErbB3	ErbB2/ErbB3	ErbB4	ErbB2/ErbB4	D23	D24	T47-14
EGF	4.5 ± 1.2	ND	-	>133	-	-	-	-
EGF/YYDLL	5.0 ± 0.7	ND	-	35 ± 15	-	-	-	-
TGFA	5.8 ± 1.2	ND	-	ND	-	-	-	-
TGFA/YYDLL	5.2 ± 0.8	ND	-	122 ± 8	-	-	-	-
T1E	7.2 ± 1.2	>35	>35	22 ± 5	4.3 ± 2.2	250 ± 45	280 ± 50	2500 ± 250
T1E/YYDLL	7.2 ± 2.8	1.1 ± 0.2	3.5 ± 0.8	2.0 ± 0.7	3.7 ± 0.7	70 ± 10	1000 ± 75	900 ± 75
EREG	33.3 ± 1.7	ND	-	83 ± 17	-	-	-	-
EREG/YYDLL	17.5 ± 4.2	35*	-	9.8 ± 3.5	-	-	-	-
NRG	ND	3.1 ± 0.7	0.7 ± 0.1	1.8 ± 0.2	0.85 ± 0.18	90 ± 10	70 ± 15	400 ± 55
NRG/YYDLL	ND	0.06 ± 0.01	0.4 ± 0.1	2.7 ± 0.7	0.88 ± 0.02	250 ± 41	140 ± 30	300 ± 50

Table I Overview of IC50 values obtained from ligand binding competition analyses and EC50 values of mitogenic assays. Levels of 50% binding competition (IC50 values) and mitogenic assays (EC50 values) were estimated graphically from the highest and lowest values experimentally obtained. ND, Not Determined. *, 35 nM is close to the IC50. -, no measurable binding affinity.

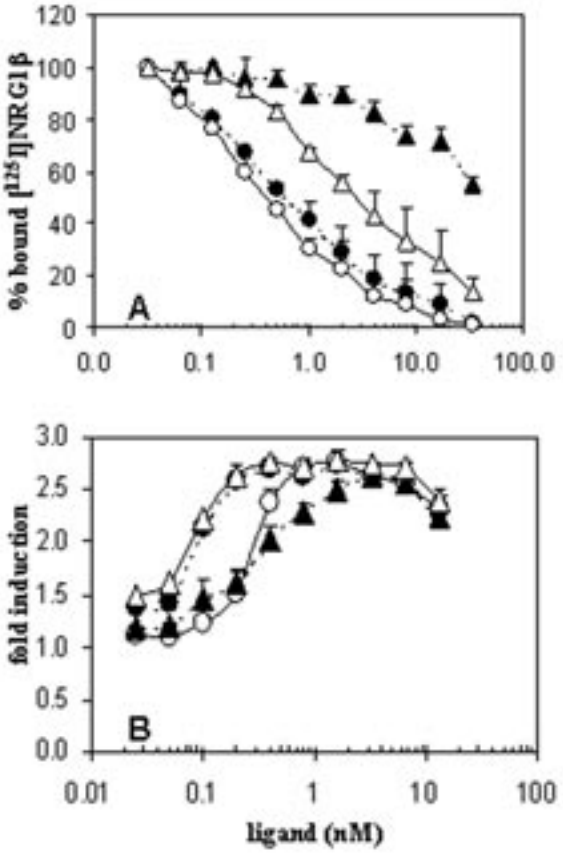

Figure 2 Binding affinity and mitogenic activity of wild-type ligands and YYDLL mutants on ErbB2-ErbB3 heterodimers
(**A**) Ligand binding displacement analysis on ErbB2 and ErbB3 overexpressing D23 cells treated with 0.17 nM [^{125}I]-NRG1β. (**B**) Dose-dependent mitogenic activation of D23 cells, measured by MTT analysis. Cells were treated with increasing concentrations of the ligands NRG1β (···●···), NRG/YYDLL (–○–), T1E (···▲···) and T1E/YYDLL (–△–). Fold induction was calculated compared to unstimulated D23 cells. Maximum stimulation corresponded to 2.78-fold induction. Results are the mean of at least two independent experiments performed in duplicate.

Increased ErbB4 affinity correlates with increased mitogenic activity

ErbB2 is known to affect both the affinity and selectivity of ligand binding to ErbB4. Figure 3A shows the effect of introducing the YYDLL sequence into NRG1β and T1E on the binding affinity towards D24 cells, which contain both ErbB2 and ErbB4. On these cells NRG1β and NRG/YYDLL are equally potent with an IC50 around 1 nM, while also T1E and T1E/YYDLL are equally potent with an IC50 around 4 nM. The relatively high affinity of T1E for ErbB2/ErbB4 heterodimers, in comparison with ErbB2/ErbB3 heterodimers (Figure 2A), thus appears to limit the effect of introducing the YYDLL sequence.

Figure 3B shows that NRG1β (EC50 70 pM) is a potent activator of ErbB2/ErbB4 heterodimers in D24 cells, but interestingly NRG/YYDLL (EC50 140 pM) appears less active. Similarly, T1E/YYDLL (EC50 1 nM) appears less active on these cells than T1E (EC50 280 pM), in spite of their similar

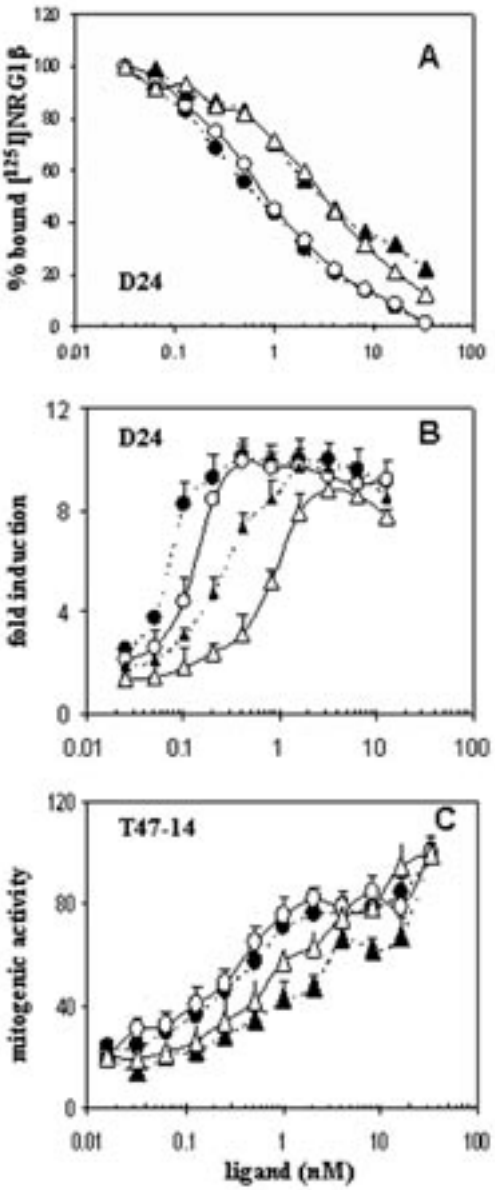

Figure 3 Binding affinity and mitogenic activity of wild-type ligands and YYDLL mutants on ErbB4 homodimers and ErbB2/ErbB4 heterodimers
(**A**) Ligand binding displacement analysis on ErbB2 and ErbB4 overexpressing D24 cells treated with 0.17 nM [^{125}I]-NRG1β. (**B**) Dose-dependent mitogenic activation of D24 cells, measured by MTT analysis. Fold induction was calculated compared to unstimulated D24 cells. Maximum stimulation corresponded to 10-fold induction (**C**) Dose-dependent mitogenic activation of T47-14 cells, measured by [^{3}H]-thymidine incorporation. Cells were treated with increasing concentrations of the ligands NRG1β (···●···), NRG/YYDLL (−○−), T1E (···▲···) and T1E/YYDLL (−△−). The highest value experimentally obtained was set on 100%. Results are the mean of at least two independent experiments performed in duplicate.

binding affinity. Unlike ErbB3, ErbB4 can also be mitogenically stimulated in the absence of ErbB2. When studying the mitogenic potential of these ligands for T47-14 cells, which contain ErbB4 in the absence of ErbB2, an opposite behaviour is seen (Figure 3C). Although it cannot be excluded that T47-14 cells contain low levels of endogenous mouse ErbB1 receptors, we have previously shown that these cells are not mitogenically stimulated by

EGF (Wingens and others 2006). Therefore the observed mitogenic effects of T1E/YYDLL on T47-14 cells must result from activation of ErbB4.

The observation that is these cells YYDLL mutants are more active, is in agreement with their increased binding affinity (EC50: NRG 0.4 nM, NRG/YYDLL 0.3 nM, T1E 2.5 nM and T1E/YYDLL 0.9 nM), as shown in Figures 1E and 1F. Interestingly, the EC50 values of NRG1β and T1E/YYDLL differ by a factor of 15 on D24 cells, but only by a factor of 2-3 on T47-14 cells. Thus, for reasons which are not well understood, ligands which share the same affinity and mitogenic activity towards ErbB4 homodimers can differ in their ability to activate ErbB2-ErbB4 heterodimers.

Homology modeling of EREG in complex with the ErbB receptors

We have previously shown that positive constraints involved in ErbB-ligand interaction can be studied from homology models of ligand-receptor complexes (van der Woning and others 2006). Such bioinformatic models have been based on the available crystal structure of TGFα-liganded ErbB1, whereby the backbone structure of TGFα has been used for all ligands and that of ErbB1 for all receptors (1). However, NMR analysis has shown that the A1-loop of EREG (region between the first and second cysteine) has a different conformation from all other mammalian EGF-like growth factors, while in addition EREG lacks the anti-parallel β-sheet that is formed by residues in the C-loop and C-terminal linear region (11,13). We have therefore investigated if this altered conformation affects the ability of EREG to interact with various ErbB receptors.

Figure 4 provides a survey of the positive constraints (electrostatic interactions, hydrophobic interactions and hydrogen bridges), which are predicted from such homology models for all combinations of ligands and receptors studied here that show detectable affinity. For ligands that do not show interaction with a specific ErbB receptor, experimentally identified negative constraints are indicated. In the case of EREG use was made of its specific NMR structure to model interaction with ErbB1 and ErbB4, while binding of EREG/YYDLL to the various ErbB receptors was modeled according to TGFα in its complex with ErbB1.

Our homology models show that EREG makes less interactions (hydrogen bounds, salt bridges or Van-der Waals interactions) with ErbB1 than the high affinity ligands EGF, TGFα and T1E (Figure 4). Although the relative contribution of each interaction site to the total binding affinity cannot be predicted by homology modeling, the lower number of interactions could explain why EREG binds with lower affinity to ErbB1 than the other ligands. Compared to EGF, EREG lacks the hydrogen bond at Asp11 (as depicted in Figure 5A), the charge interaction of Lys28, the hydrogen bond of Glu40, and the hydrophobic interaction of Tyr44 (EGF numbering is used throughout the manuscript). However, the lower number of interaction sites does not seem to

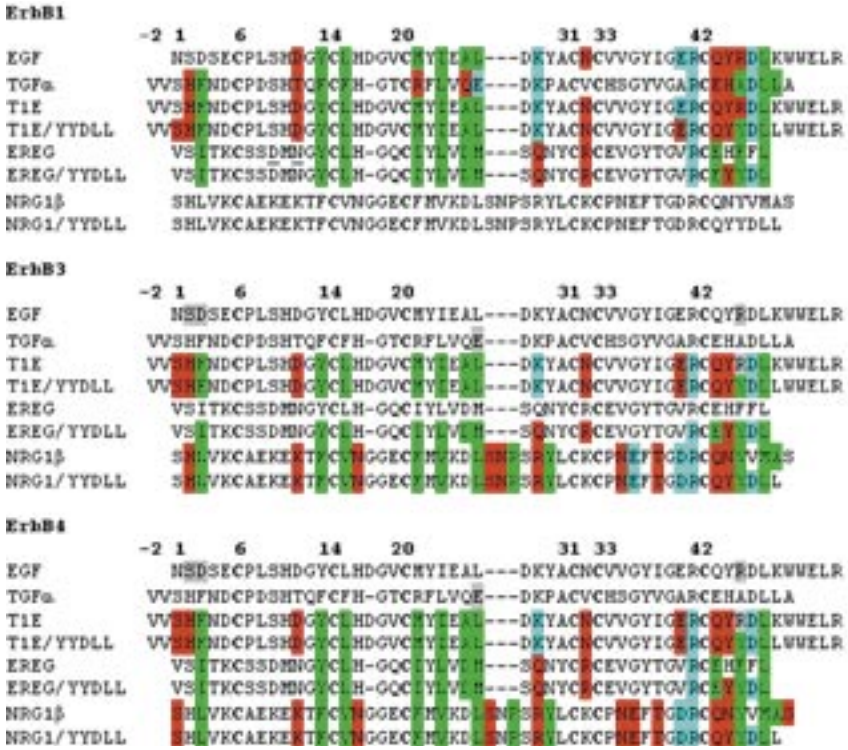

Figure 4 Interaction sites for binding of EGF-like growth factors to ErbB1, ErbB3 and ErbB4, based on bioinformatics homology models

Presented sequences are of human EGF, TGFα , T1E, EREG and NRG1β, as well as of their YYDLL mutants. Conserved cysteines are numbered and are depicted in bold. EGF numbering is used throughout this study. Residues in red are involved in hydrogen bond formation, residues in green are involved in hydrophobic interactions, and residues in blue are involved in salt bridge formation with the indicated ErbB receptors. For ligands lacking ErbB interaction, negative constraints are depicted in grey, when known. Different ligands may share a residue on the same position in the linear representation, of which the one interacts with a receptor and the other does not, because of differences in rotamer positions of the amino acids determined by the surrounding sequences.

result from the specific conformation of EREG, since similar interactions were observed for residues 1-46 of EREG when modeled according to its own NMR structure (EREG) and according to TGFα (EREG/YYDLL).

Introduction of the YYDLL sequence into EREG provides additional interaction sites with ErbB1 (Figure 4), but our experimental data show that this results in only a small increase in binding affinity for this receptor (Figure 1A,B). The observation that introduction of additional positive constraints into a ligand with moderate ErbB1 affinity does not increase this binding affinity,

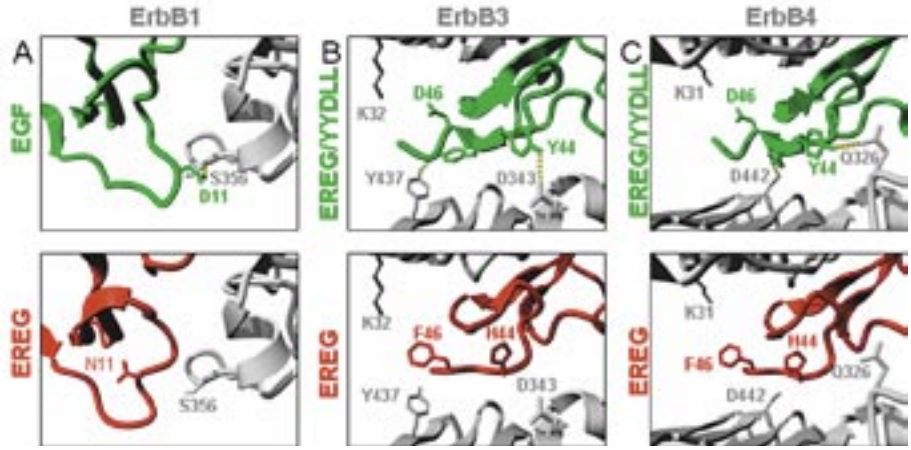

Figure 5 Homology models for the structure of EREG, EREG/YYDLL and EGF in complex with the extracellular domains of ErbB1, ErbB3 and ErbB4
Close-up pictures of ribbon representations are shown for the involvement of (**A**) the A1 loop of EGF and EREG upon ErbB1 interaction, (**B**) interaction of the C-terminal linear region of EREG/YYDLL and EREG with ErbB3, (**C**) idem with ErbB4. ErbB receptors are depicted in grey, EGF and EREG/YYDLL in green and EREG itself in red. Yellow dotted lines represent hydrogen bounds. The amino acid numbering of the ligands is according to that of EGF, and the amino acid numbering of the receptors is according to Jorissen *et al.* (26). Rotamers of the amino acid side chains were positioned using SCWRL (16), refined in YASARE and checked with WHAT IF(18) quality control indicators.

suggests that the affinity of EREG for ErbB1 is limited by the presence of so far unidentified negative constraints outside the C-terminal linear region, which may result from the aberrant conformation of EREG. Domain-exchange mutants between EGF and EREG will have to indicate to what extent the aberrant conformation of EREG in the A1- and C-loop is indeed responsible for its relatively low affinity for ErbB1.

The observation that the affinity of EREG for ErbB3 and ErbB4 is strongly enhanced upon introduction of the YYDLL sequence in the C-terminal linear region, suggests that for binding to these receptors the number of positive constraints in EREG is limiting its affinity. This is illustrated in Figures 5B and 5C, which show that additional interaction sites are formed by Tyr44, Tyr45 and Asp46 in EREG/YYDLL upon binding to ErbB3 and ErbB4 respectively. The possibility cannot be excluded, however, that the YYDLL sequence does not only enhance the number of positive constraints in EREG, but also relieves a sofar unidentified negative constraint in the C-terminal linear region of this ligand, similar to our previous observation for EGF binding to ErbB3 and ErbB4 (van der Woning and others 2006). Our homology models do not provide any indication, however, that His44 and Phe46 may be involved in any such negative constraint (Figures 5B and 5C).

DISCUSSION

In present study we have investigated the role of the C-terminal linear region of EGF-like growth factors in their binding affinity and selectivity for ErbB receptors. We have previously shown that the C-terminal sequence YYDLL is compatible with binding to all three ligand binding ErbB receptors (van der Woning and others 2006). Here we have shown that introduction of the YYDLL sequence has only minor effects on the binding affinity of ligands for ErbB1, but strongly enhances the binding affinity of NRG1β for ErbB3. Furthermore, introduction of the YYDLL sequence can alter receptor specificity, as a result of which TGFα gains affinity for ErbB4 and EREG for ErbB3. For reasons that are not well understood within the current models of ErbB-ligand interaction, NRG/YYDLL showed a much higher affinity for binding to ErbB3 than to ErbB2/ErbB3 heterodimers. Furthermore the mitogenic activity of ligands for ErbB2/ErbB3 did not parallel their binding affinity for these heterodimers. In contrast, the mitogenic activity of ligands for ErbB4 homodimers and ErbB2/ErbB4 heterodimers largely paralleled their binding affinity. In combination these data show that the C-terminal linear region of EGF-like growth factors plays a crucial role in ErbB affinity and selectivity.

We have previously postulated that EGF-like growth factors contain a limited number of residues that are not essential for high affinity receptor binding, but that prevent the unintended binding to other ErbB receptors. These so-called negative constraints thereby form the molecular basis for the observed ligand specificity of receptor binding. In previous studies we have shown that binding to ErbB3 is strongly impaired by the ligand residues Glu26 (in the B-loop) and Arg45 (in the C-terminal linear region), a well as by the absence of beta-sheet forming residues in the linear N-terminal region. Based on our present and previous studies, we propose that the main difference in binding requirement between ErbB3 and ErbB4 is located in the ligand's N-terminal linear region. This is indicated by the observation that introduction of a WVR sequence into the N-terminal linear region allows the mutant WVR/EGF/IADIQ to bind to ErbB1 and ErbB3, but not to ErbB4 (21).

It has been postulated that during evolution a single parental ErbB gene has been segregated into an ErbB1/ErbB2 and an ErbB3/ErbB4 ancestor gene. Activation of those receptors would require a TGFα-type ErbB1 specific ligand and a NRG-type ErbB3/ErbB4 specific ligand. The observation that no natural ligand exists that binds both ErbB1 and ErbB3, suggests that known ErbB1/ErbB4 bispecific ligands (BTC, EREG, HB-EGF) developed after a second whole genome duplication which resulted in the generation of four different ErbB genes. It is speculative whether during this evolutionary process some ErbB1-specific ligands gained affinity for ErbB4, or that some ErbB1/ErbB4 bispecific ligands lost affinity for ErbB4. In our view on the role of negative constraints in ligand binding specificity, the second option seems more feasible, since it would require only a few inactivating mutations to release an existing negative constraint for ErbB4. Still there appears to have been a

strong evolutionary pressure to generate and maintain ErbB1/ErbB4 bispecific ligands, since the TGFα/BTC, EPGN/EREG and AREG/HBEGF ancestors have all three diversified to both an ErbB1 specific ligand and an ErbB1/ErbB4 bispecific ligand.

Many studies have been performed on the structure-function relationship of the ErbB1 specific ligands EGF and TGFα, but much less is known about the requirements for binding of NRGs to ErbB3 and ErbB4. Alanine scanning mutagenesis and phage display affinity optimisation studies have shown that the requirements of NRG1β for binding to ErbB3 and ErbB4 partly overlap, but are not identical (2,22). Still it has proven impossible sofar to design NRG mutants that specifically bind to either ErbB3 or ErbB4 (23). In contrast, mutants that are specific for either ErbB3 or ErbB4 can readily be made on the basis of the primary structure of EGF, although always concomitant with high affinity for ErbB1 (21).

Crystal structure analysis has indicated that ErbB dimerisation stabilizes high affinity ErbB/ligand complexes. Such complexes can either consist of two liganded ErbB receptors, or a single liganded ErbB receptor in complex with the orphan ErbB2. Our current observation that NRG/YYDLL binds with much higher affinity to cells containing only ErbB3 than to cells containing both ErbB2 and ErbB3 is therefore puzzling. Possibly, NRG/YYDLL can avidly bind to putative pre-existing ErbB3 homodimers on the cell surface. However, the ability of ErbB3 to form homodimers has been disputed, particularly based on experimental studies with the soluble extracellular domain of ErbB3 (24). Alternatively, the affinity of NRG/YYDLL for ErbB3 monomers may be so high, that no further gain of affinity is observed upon receptor dimerisation.

In contrast to ErbB3, the affinity of NRG1β for ErbB4 could not be improved by introduction of the YYDLL sequence. This must imply that either the affinity of NRG1β for ErbB4 is already optimal and cannot be further enhanced by mutations, or that the C-terminal linear region of this ligand does not provide an important contribution to ErbB4 binding. However, various natural isoforms of the NRGs exist which differ in their C-terminal linear region. These isoforms differ strongly in their binding affinity for ErbB3 and ErbB4. Particularly for NRG2 it has been shown that Lys45 in the C-terminal linear region of the low affinity isoform NRG2β impairs high affinity binding to ErbB4 (25). This residue is absent in the high affinity isoform NRG2α and therefore acts as a negative constraint for this receptor. On the other hand various studies, including the present one, have shown that the affinity of NRG1β for ErbB3 can be enhanced 10-100 fold by directed mutations in the B-loop and C-terminal linear region (2). This implies that during evolution EGF-like growth factors may not have evolved for optimal binding affinity, particularly not for binding to ErbB3. It remains unclear, however, why low affinity EGF-like growth factors, such as EREG for ErbB1, and NRG1α and NRG2β for ErbB3 and ErbB4, are maintained during evolution.

In the case of ErbB3 it could be argued that high affinity ligands will favour the formation of inactive ErbB3 homodimers, while low affinity ligands

will favour the formation of biologically active ErbB2/ErbB3 heterodimers. This hypothesis is strengthened by our observation that NRG/YYDLL, with its very high binding affinity for ErbB3 alone, shows lower mitogenic activity than NRG1β on cells containing both ErbB2 and ErbB3. Moreover, introduction of YYDLL enhances the binding affinity of T1E for ErbB2/ErbB3 heterodimers, although the affinity is still lower than that of NRG1β. Still, in a mitogenic assay T1E/YYDLL shows equally high activity as NRG1β. Therefore it appears that the natural ligand NRG1β combines an optimal binding affinity for ErbB3 with the highest mitogenic activation of ErbB2/ErbB3 heterodimers.

ACKNOWLEDGMENTS

We thank Cathelijne Frieling and Annemiek Eek (Department of Nuclear Medicine, Radboud University Nijmegen Medical Center) for radiolabeling of the growth factors. The present work was supported by grants from the Netherlands Organization for Scientific Research (NWO-CW), The Netherlands Cancer Society, and the Stichting Bergh in het Zadel (Beek, The Netherlands).

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6

Quantification of ErbB3 receptor density on human breast cancer cells, using a stable radiolabeled mutant of NRG1β

Biochemical Biophysical Research Communications 378, 285-289 (2009)

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ABSTRACT

ErbB3 transactivation can make tumor cells resistance to ErbB1/ErbB2 targeting drugs. This urges for a reliable method to determine cell surface ErbB3 levels, but in our hands iodinated NRG1β is unstable and tends to underestimate the number of ErbB3 receptors in a radio-receptor assay. Here we show by the use of a radio-labeled high affinity neuregulin mutant NRG/YYDLL that ErbB3 levels can be determined in a reliable manner by Scatchard analysis. Furthermore we show by differential competition with unlabeled NRG/YYDLL and betacellulin that the number of ErbB3 and ErbB4 receptors can be quantified separately on cultured human breast cancer cells.

INTRODUCTION

The ErbB signaling network is currently one of the main targets for the development of anti-cancer drugs. The ErbB family of type 1 transmembrane receptor tyrosine kinases consists of four members: ErbB1 (EGFR), ErbB2, ErbB3 and ErbB4. ErbB1 and ErbB2 are frequently overexpressed in epithelial cancers, where they are involved in autonomous cell proliferation, invasion and metastasis. In recent years FDA-approved drugs have been developed, including ErbB1-specific tyrosine kinase inhibitors (erlotinib, gefitinib) and ErbB specific antibodies (cetuximab, trastuzumab), which are widely used in treatment of e.g. breast, lung, colorectal and head-and-neck cancers [1]. Although such treatments can be very successful, their long term application is limited by the observation that many patients may eventually develop resistance to applied drugs [2; 3].

Recent data show that activation of ErbB3 provides one of the mechanisms whereby tumor cells can escape from ErbB1 and ErbB2-directed therapy [4]. ErbB3 has an impaired tyrosine kinase domain, but after binding of its endogenous ligand NRG1β it can form strongly active heterodimers with ErbB2, and to a lesser extent also with ErbB1 or ErbB4 [5]. ErbB3 is frequently overexpressed in breast cancers, where it can be transactivated by the concomitantly overexpressed ErbB1 and ErbB2. Recent data also show that ErbB2 and ErbB3 are both more active in cetuximab-resistant than in cetuximab-responsive non-small cell lung carcinoma cells, indicating that other receptors than ErbB1 contribute to the resistance of tumor cells for anti-ErbB1 therapies [6]. Moreover, it has been shown that direct targeting of ErbB3 by siRNA in resistant cells restores cetuximab sensitivity to these cells [6]. This observation suggests that ErbB3 may sequester ErbB1 into a biologically active ErbB1/ErbB3 complex, which is insensitive to inhibition by

cetuximab.

These observations indicate that determination of ErbB3 levels in tumor tissue may be critical in the selection of patients for ErbB-directed therapy, as well as for their prognostic potential. ErbB receptor densities can be determined at the mRNA level by quantitative PCR analysis or fluorescence in situ hybridization, and at the protein level by flow cytometric analysis or immunoblotting using receptor specific antibodies. However, these methods do not readily allow quantification of receptor numbers. The density of ErbB1 receptors in both intact cells and cellular extracts is routinely determined by a radio-receptor assay employing the ErbB1-specific ligand epidermal growth factor (EGF) as radiolabeled [^{125}I] ligand. NRG1β is a high affinity ligand for both ErbB3 and ErbB4, but binding studies using [^{125}I]-NRG1β are scarce in the literature [7; 8]. One of the reasons might be that, according to our experience, NRG1β rapidly loses its high binding affinity for ErbB receptors following iodination, possibly due to sulfoxidation of methionine residues. In contrast to EGF, NRG1β contains a methionine (residue 47 according to EGF numbering) at a position which is essential for high affinity ErbB3 binding. Although direct experimental evidence is lacking, we propose that the observed instability of NRG1β upon iodination results from sulfoxidation of Met47.

We have recently shown that exchange of the five most C-terminal residues of NRG1β into the sequence YYDLL enhances its binding affinity for ErbB3. In the present study we show that with NRG/YYDLL as radioligand 5-10 fold higher bound/added values are detected on ErbB3 containing cells than with [^{125}I]-NRG1β, resulting in significantly higher receptor numbers and lower Kd-values in Scatchard plots. Using this radioligand we were subsequently able to quantify the number of ErbB3 and ErbB4 receptors on a set of cultured human breast cancer cell lines.

MATERIALS AND METHODS

Cell lines

Murine 32D hematopoietic progenitor cells transfected with distinct human ErbB-encoding plasmids (ErbB3 for D3 cells; both ErbB1 and ErbB3 for D13 cells) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Paisley, UK) and 0.25 ng/ml murine interleukin-3 (Promega, Madison, WI), and were subsequently kept under continuous selection using 0.6 mg/ml G418 (Calbiochem, La Jolla, CA). Human breast cancer cell lines T47D and MCF7 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS) (Perbio, Logan, UT). CAMA1 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS.

Radioligand displacement analysis

Human NRG1β (R&D Systems, Mineapolis, MN) and its mutant NRG/YYDLL (Van der Woning et al., submitted) were iodinated enzymatically to a specific activity of approximately 1.2 Ci/μmol [9]. For ligand displacement studies on human breast cancer cells, $5.0x10^5$ T47D, MCF7 and CAMA1 cells were seeded in 24-wells plates in 1 ml of DMEM/NCS. After 2 days of culture, cells were incubated for 2 hours on ice in 100 μl of HEPES-buffered DMEM/NCS, containing 5 ng/ml of [^{125}I]-NRG/YYDLL in additional presence of serial dilutions of unlabeled NRG/YYDLL or betacellulin (R&D Systems). Cells were subsequently lysed in 0.5 ml of a 1% Triton X-100 solution, and the bound radioactivity was measured by γ-counting. Scatchard analysis binding studies on D3 and D13 cells were performed in suspension, whereby $5.0x10^6$ cells were suspended in 150 μl of HEPES-buffered RPMI/FCS, supplemented with 1 ng/ml of radiolabeled ligand. After incubation for 2 hours on ice, cells were washed twice with serum-free RPMI medium, and spun down for 20 seconds at 13,000 rpm after which bound radioactivity was measured by γ-counting. Radioligand displacement curves were analyzed following the linear subtraction method [10] and data for specific binding subsequently presented in a Scatchard plot.

Western blotting

For immunodetection of ErbB3 receptors, $1.0x10^6$ D1 or D13 cells cells were lysed in RIPA buffer supplemented with proteinase inhibitors aprotein, leupeptin, pepstatin and PMSF (Sigma, St. Louis, MO). Following gel electrophorsis, immunoblotting was performed using anti-ErbB3 antibodies from Cell Signaling (Beverly, CA).

Flowcytometric analysis

For determination of ErbB3 expression level on 32D cells, $5.0x10^5$ cells were Fc-blocked by treatment with 1 μg of human IgG/10^5 cells for 15 minutes at room temperature and subsequently stained for 1 hour with 5 μg/ml phycoerythrin-labeled anti-ErbB3 antibodies (R&D Systems) in the same buffer. Cells were then washed twice and resuspended in ice-cold PBS/1%NCS before fluorescence was analyzed at 488 nm on the FACScan system (Beckman Coulter, Fullerton, CA).

Real-time quantitative PCR

mRNA levels for ErbB3 were determined following TriZol (Invitrogen) treatment of $1.0x10^6$ 32D cells, according manufacturer's protocol. cDNA was produced from 1 µg of RNA using Superscript Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was performed on the 7500-real-time PCR system from Applied Biosystems and data were analyzed using 7500 SDS software. mRNA levels were expressed relative to that of the household gene GAPDH.

RESULTS

Quantification of ErbB3 receptor levels by ligand binding analysis

Following NRG1β binding, ErbB3 can form stable heterodimers with the orphan receptor ErbB2, but there is increasing evidence that active complexes can also be formed with unliganded ErbB1 receptors [11]. In the present study we have determined the binding affinity of NRG1β and its mutant form NRG/YYDLL for cells containing only ErbB3 (D3 cells) or both ErbB1 and ErbB3 (D13 cells) receptors. Figure 1A shows ligand binding displacement curves for both cell lines, using either 1 ng/ml of freshly labeled [^{125}I]-NRG1β or [^{125}I]-NRG/YYDLL as radioligand. The data show that D13 cells bind 1.5-2.5 times more labeled ligand than D3 cells, while in the case of [^{125}I]-NRG/YYDLL 4.5-7.5 times more radioactivity was bound than with [^{125}I]-NRG1β as labeled ligand. In all cases the binding of labeled ligand was fully competed by addition of a 200-fold excess of either unlabeled NRG1β or NRG/YYDLL.

Figure 1B shows the corresponding Scatchard plots for specific receptor-ligand binding. The Scatchard plots all show a linear behavior, indicating that within the range of ligand concentrations used there is no evidence for the presence of both high and low affinity sites, as is generally observed for ligand binding to ErbB1 [12]. Figure 1C gives a survey of the apparent ligand binding dissociation constants (K_d) and ErbB3 receptor densities, obtained from these Scatchard plots. The data show that using [^{125}I]-NRG/YYDLL as a label 5-10 fold higher levels of ErbB3 receptors/cell were observed than with [^{125}I]-NRG1β, concomitant with a 3-fold lower Kd-value. The enhanced affinity of NRG/YYDLL for ErbB3 may result from the introduced mutations in the C-terminal linear region, but the much higher ErbB3 levels identified by NRG/YYDLL binding most likely indicates that the actual receptor numbers are underestimated when NRG1β is used as a labeled ligand. The observation that binding of [^{125}I]-NRG/YYDLL can be fully competed by an excess of unlabeled NRG1β (data not shown) implies that NRG/YYDLL does not bind to additional ErbB3 binding sites compared to NRG1β. These data are therefore best understood if it is assumed that under the conditions tested some 80-

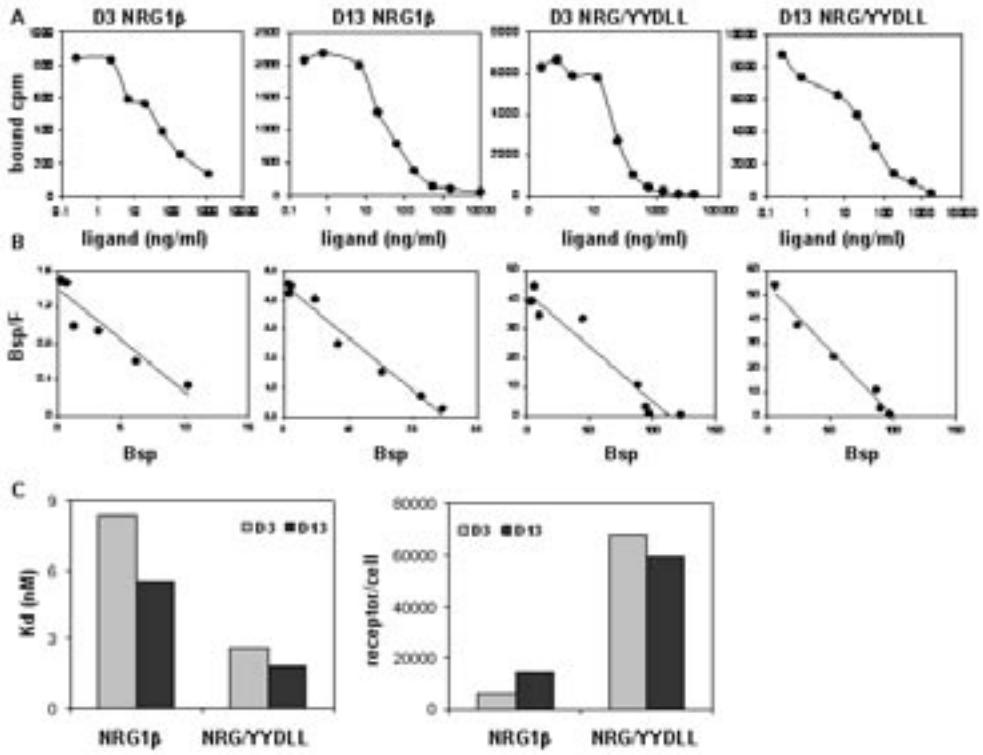

Figure 1. Determination of the ErbB3 receptor levels on 32D cells. (A) Ligand binding displacement curves on D1 and D13 cells, treated with 1 ng/ml of the indicated labeled ligand and increasing concentrations of the same unlabeled ligand. (B) Scatchard plots for NRG1β and NRG/YYDLL binding to D1 and D13 cells, each underneath its corresponding displacement curve. Specific ligand binding (Bsp) has been expressed in fmoles/10^6 cells, and free ligand concentration (F) in nM. (C) Survey of apparent ligand binding affinities and receptor numbers obtained from Scatchard analysis for D3 (grey) and D13 (black) cells. Bound/added values of labeled ligand were: 4% (D3-NRG1β); 10% (D13-NRG1β); 49% (D3-NRG/YYDLL) and 69% (D13-NRG/YYDLL). Data Presented are the average of two independent experiments in duplicate.

90% of the labeled NRG1β is no longer biologically active and unable to bind ErbB3 with high affinity, possibly due to sulfoxidation of Met47. Moreover, no significant difference in Kd was observed for ligand binding to D1 and D13 cells, indicating that heterodimerization with ErbB1 does not appear to enhance the affinity of NRG1β for its native receptor.

Comparison of ErbB3 levels on D1 and D13 cells by flowcytometric analysis, Western blotting and real-time quantitative PCR

The above binding studies in which [^{125}I]-NRG1β was used as a radiolabeled ligand, suggest that D13 cells may contain twice the number of ErbB3 cell surface receptors as D3 cells, while no such difference in receptor density was observed when [^{125}I]-NRG/YYDLL was used as radioligand. Subsequent flow cytometric analysis (Fig.2A) of cells labeled with anti-ErbB3 antibodies showed a similar cell surface receptor density on D3 and D13 cells, while as a negative control D12 cells (containing exclusively ErbB1 and ErbB2) were devoid of cell surface staining. Although receptors cannot readily be quantified by this technique, the data confirm the results obtained from the [^{125}I]-NRG/YYDLL binding studies. Quantitative real time PCR analysis

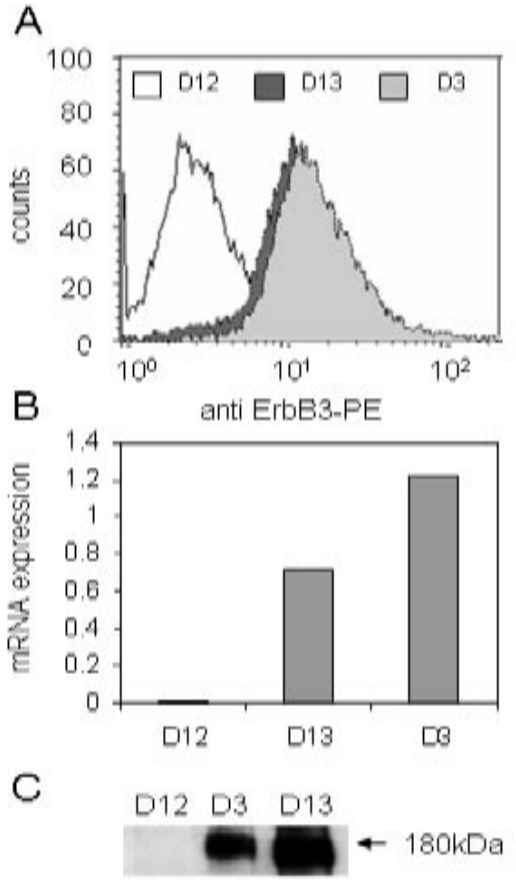

Figure 2. Determination of the ErbB3 expression level in 32D cells. (A) Flow cytometric analysis of ErbB3 levels, using a PE-conjugated anti-ErbB3 antibody, on D12 (white), D3 (grey) and D13 (black) cells. (B) ErbB3 mRNA levels, determined by quantitative PCR and expressed relative to GAPDH. (C) Western blotting analysis of ErbB3 levels in whole cell lysates, detected by anti-ErbB3 antibodies.

(Fig. 2B) showed that the ErbB3 mRNA level is 1.7-fold higher in D3 cells than in D13 cells, while Western blot analysis (Fig. 2C) indicated that whole cell lysates of D13 cells contain at least twice the amount of ErbB3 protein present in D3 cells. These data indicate that ErbB3 mRNA levels cannot directly be related to protein levels. Moreover, it appears that significant amounts of ErbB3 receptors may be located intracellularly, where they cannot be reached by extracellularly added ligands or antibodies.

Discrimination between NRG/YYDLL binding to ErbB3 and ErbB4

The present study indicates that binding studies with radiolabeled NRG/YYDLL provide a robust method for quantifying ErbB receptor densities, but it should be taken into account that NRG/YYDLL binds with high affinity to both ErbB3 and ErbB4, albeit not to ErbB1. Figure 3 shows that ErbB3 specific binding can be quantified when [^{125}I]-NRG/YYDLL binding to cells is not only competed by increasing concentrations of unlabeled NRG/YYDLL, but in parallel also with unlabeled betacellulin (BTC), an EGF-like growth factor that binds both ErbB1 and ErbB4, but not ErbB3. Unlabeled NRG/YYDLL will compete for binding of [^{125}I]-NRG/YYDLL to both ErbB3 and ErbB4, while BTC will compete only for binding to ErbB4. When applied to human breast cancer cell lines in tissue culture using a high concentration (5 ng/ml) of [^{125}I]-NRG/YYDLL to assure at least 50% receptor occupancy, it is shown in Fig. 3 that on T47D cells 40% of bound labeled ligand could be competed with BTC and

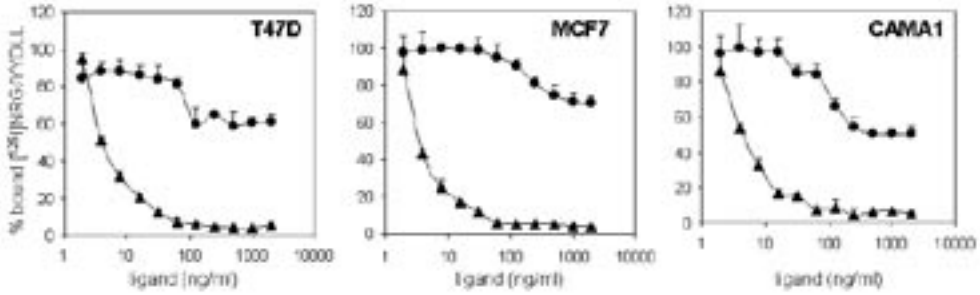

Figure 3. Determination of the ErbB3 and ErbB4 levels on human breast cancer cells. Binding of 5 ng/ml [^{125}I]-NRG/YYDLL was competed by unlabeled NRG/YYDLL (closed triangles) or BTC (closed circles). Bound/added values of labeled ligand were 5% (T47D), 2.5% (MCF7), and 5% (CAMA-1). Data presented are the average of two independent experiments in duplicate.

100% by NRG/YYDLL, indicating that the 60% of the NRG1β binding sites are ErbB3 and 40% ErbB4. Similarly, MCF7 cells contain 70% binding sites for ErbB3 and 30% for ErbB4, while CAMA-1 cells have a similar density of ErbB3 and ErbB4 receptors. Based on the total amount of [^{125}I]-NRG/YYDLL bound, this corresponds to $1.5x10^4$ ErbB3 and $1.0x10^4$ ErbB4 receptors on T47D cells; $8.75x10^3$ ErbB3 and $3.75x10^3$ ErbB4 receptors on MCF7 cells; and $1.25x10^4$ ErbB3 and $1.25x10^4$ ErbB4 receptors on CAMA-1 cells.

DISCUSSION

There is increasing interest in the role of ErbB3 in tumorigenesis. ErbB3 is overexpressed in many epithelial tumors, but since ErbB3 lacks intrinsic tyrosine kinase activity, drug design has mainly been focussed on ErbB1 and ErbB2. The recent observation that high ErbB3 expression levels can make tumor cells resistant to ErbB1-directed tyrosine kinase inhibitors [4], urges for a reliable method to quantify ErbB3 levels on the cell surface. In the present study we have shown that a radiolabeled mutant of neuregulin (NRG/YYDLL) provides a stable and reliable ligand for quantification of both ErbB3 and ErbB4 levels on the surface of human breast cancer cell lines.

Although ErbB3 lacks tyrosine kinase activity, it can be tyrosine phosphorylated by active ErbB1 and ErbB2 receptors, which results in a strong activation of particularly the PI3K-pathway. It is well established that upon NRG1β binding ErbB3 is able to bind ErbB2, upon which both receptors become tyrosine phosphorylated [13; 14]. Moreover, we and others have shown that, upon NRG1β binding, ErbB3 can also form a mitogenic complex with ErbB1, even in the absence of an ErbB1 binding ligand such as EGF [11]. It has been speculated that a small fraction of unliganded ErbB1 receptors in the so-called open conformation is involved in this ErbB1/ErbB3 heterodimer formation [15]. It is currently unclear if this fraction of ErbB1 receptors is sensitive to the tyrosine kinase inhibitors erlotinib and gefitinib. It is also unclear if ErbB3-mediated resistance to these inhibitors requires the presence of NRG1β. Interestingly, ErbB1/ErbB3 complexes are readily dissociated in the presence of an ErbB1 activating ligand [11]. It can therefore be anticipated that cetuximab, which prevents ligand binding to ErbB1, may stabilize the activity of such ErbB1/ErbB3 heterodimers. Determination of the exact number of ligand binding ErbB3 molecules on tumor cells is a first step towards our understanding of ErbB3-mediated resistance to ErbB1 and ErbB2 targeting drugs. This information will be very important for choosing the proper therapy for treatment of ErbB overexpressing cancers and will predict the risk of escape from ErbB1 and ErbB2-targeted therapies.

ACKNOWLEDGEMENTS

We thank Cathelijne Frieling and Annemiek Eek (Department of Nuclear Medicine, Radboud University Nijmegen Medical Center) for radiolabeling of the growth factors. The present work was supported by grants from the Netherlands Organization for Scientific Research (NWO-CW), The Netherlands Cancer Society, and the Stichting Bergh in het Zadel (Beek, The Netherlands).

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7

Why does NRG1β bind exclusively to ErbB3 and ErbB4, and not to ErbB1?

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ABSTRACT

ErbB ligands bind their receptors in a highly specific manner. In previous reports we demonstrated that negative constraints in EGF and TGFA prevent the interaction with ErbB3 and ErbB4. Still it is unknown why NRG1β does not bind ErbB1. In this chapter we attempted to answer this question by a bioinformatics approach in which the amino acid interactions between EGF and TGFA with ErbB1 were compared with the interactions between NRG1β with ErbB3 and ErbB4. Although there are some minor differences, NRG1β-ErbB3/ErbB4 interaction is very similar to EGF/TGFA-ErbB1 interaction. Furthermore, based on these comparisons no indication was obtained for steric clashes or charge repulsions between NRG1β and ErbB1. Experimental domain exchange studies between panerbin and NRG1β showed that the A1-loop and C-loop of NRG1β contain sequences that impair ErbB1 interaction. It remains unclear, however, by what mechanism the A1 and C-loop of NRG1β prevent ligand interaction with ErbB1.

INTRODUCTION

The ErbB signaling network is highly conserved in multicellular organisms and has evolved from a single receptor-ligand complex, as is still present in invertebrates such as *C. elegans,* to a complex system consisting of multiple ErbB receptors and a variety of EGF-like ligands in mammals (Van Leeuwen, unpublished). The high degree of receptor specificity of EGF-like ligands allows the ErbB signaling network to play an important role in many distinct developmental processes. The four mammalian receptors, ErbB1, ErbB2, ErbB3 and ErbB4, bind a total of eleven different ligands. The ligands epidermal growth factor (EGF), transforming growth factor-alpha and heparin-binding epidermal growth factor (HB-EGF) bind exclusively to ErbB1, while amphiregulin (AREG), betacellulin (BTC) and epiregulin (EREG) bind both ErbB1 and ErbB4. The large family of neuregulins (NRG1-4) with their multiple splice variants binds exclusively to ErbB3 and ErbB4. There is great interest in understanding the specificity of receptor-ligand interactions. The multiple players in the ErbB network do not only allow an understanding why certain ligands bind their specific receptor, but also why they do not bind to highly related receptors.

Although the specificity of natural EGF-like ligands for binding to ErbB receptors is high, we and others have shown that a limited number of mutations in ligand molecules is sufficient to broaden their ErbB receptor specificity. EGF binds exclusively to ErbB1, but exchange of its N-terminal linear region with that of NRG1β (biregulin) or TGFA (T1E) is sufficient to make EGF also a low affinity ligand for ErbB3 and ErbB4 (1). In fact, introduction of only two residues from the N-terminal linear region of TGFA into EGF (S2H/D3F) is sufficient to make EGF a ligand that binds multiple ErbB receptors (2).

A different observation was made when intercysteine regions are exchanged between EGF and NRG1β. Introduction of a single intercysteine region of EGF into NRG1β leaves the latter's affinity for ErbB3 and ErbB4 intact, but does not result in detectable affinity for ErbB1 (1). In contrast, introduction of a single intercysteine region of NRG1β into EGF strongly reduces the affinity for ErbB1, without a detectable gain in affinity for ErbB3 and ErbB4(1).

Crystal structure analysis of liganded and unliganded ErbB receptors have provided detailed information about the requirements for receptor-ligand binding. Unliganded ErbB1, ErbB3 and ErbB4 have a highly similar conformation, in spite of considerable differences in their primary structure. EGF-like ligands have a very tight conformation, due to the presence of three disulphide bridges, which is not significantly altered upon receptor binding. Crystal structures of the EGF/ErbB1 and TGFA/ErbB1 complexes have provided detailed information about the ligand residues that directly contact their receptor (3, 4). These residues, collectively referred to as positive constraints, directly contribute to high affinity receptor binding by a combination of hydrogen bridges, electrostatic and hydrophobic interaction (2, 5). Based on these experimental structures we have subsequently derived homology models for a variety of other ligands in complex with ErbB1, in particular for the artificial ligand panerbin (T1E/YYDLL) which binds all three ligand-binding ErbB receptors with high affinity (5).

No such crystal structures have been obtained yet for NRG1β alone or in complex with ErbB3 or ErbB4. As a result much less is known about the interaction of ligands with these two receptors. An NMR structure of NRG1α has been published, which shows high similarity with the three-dimensional conformation of EGF and TGFA (6). In the present study we have made a homology model for binding of NRG1β to ErbB3 and ErbB4, based on the the published TGFA/ErbB1 crystal structure. Using this approach putative interaction sites between NRG1β and its receptors could be identified. These potential positive constraints in NRG1β were highly similar to the ones identified for EGF and TGFA upon binding to ErbB1. The observation that NRG1β cannot bind ErbB1 does therefore not appear to result from a lack of positive constraints, but more likely from the presence of residues that impair binding to ErbB1 as a so-called negative constraint. To experimentally identify such negative constraints, we have tested in this study a set of chimeras between NRG1β and panerbin for binding to ErbB1. NRG1β binds exclusively to ErbB3 and ErbB4, while panerbin binds in addition to ErbB1. Our data show that introduction of particularly the A1-and C-loop of panerbin allows NRG1β to bind ErbB1, although with low affinity, without impairing its affinity for ErbB4.

MATERIALS AND METHODS

Cell Lines

HER-14 cells and T47-14 cells were cultured in gelatinized flasks in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (12).

Expression and Purification of Mutant Growth Factors

Recombinant EGF-like growth factors were expressed as protein A-tagged fusion proteins in the protease K-deficient *Escherichia coli* strain KS474 and isolated from the periplasmic space as described (7). Growth factors were purified by means of affinity chromatography using IgG-Sepharose, followed by Factor-X cleavage, an additional round of affinity chromatography to remove the protein A-tag and a final reverse phase (RP)-HPLC purification step to remove disulphide bridge mismatches. The amount of growth factor was calculated from the peak area (absorption at 229 nm) in the RP-HPLC chromatogram, using natural mEGF as a standard. The affinity for ErbB1 was measured in an [^{125}I]-mEGF binding competition assay on HER14 cells, and for ErbB4 in an [^{125}I]-NRG1β binding competition assay on T47-14 cells, respectively.

Ligand Binding Displacement Experiments

Recombinant EGF and NRG1β were radiolabeled enzymatically to a specific activity of 1.1 Ci/μmol (8). Ligand binding displacement analyses were performed either using a suspension of 5.0×10^6 32D cells or confluent monolayers of HER14 or T47-14 cells. 32D cells were washed once with RPMI supplemented with 0.5% BSA and subsequently incubated for 2 hours at 4^{o}C with serial dilutions of unlabeled ligand in the presence of 1 ng/ml of [^{125}I]-NRG1β. Cells were then washed once with binding buffer and loaded onto a serum cushion to remove the unbound label. Subsequently, cells were quickly centrifuged at 2000 rpm, and cell surface-bound radioactivity was determined by γ-counting. HER14 or T47-14 cells were grown to confluency, and serial dilutions of unlabeled growth factors were added in the presence of 1 ng/ml of [^{125}I]-mEGF or 1 ng/ml of [^{125}I]-NRG1β, respectively. After incubation for 2 hours at 4 ^{o}C, the cells were washed three times with phosphate-buffered saline (PBS) in order to remove the unbound label and subsequently incubated for 1 hour in 1% Triton X-100 at room temperature prior to γ-counting.

Homology Modeling of NRG1β in Complex with ErbB3 and ErbB4

Homology models were constructed for the extracellular domains of ErbB3 and ErbB4, all in complex with NRG1β. Initial models were built using both the ErbB1-EGF complex (3) and the ErbB1-TGFA complex as a template. Validation of the models with WHAT CHECK (9) identified template 1MOX, the ErbB1-TGFA complex solved at a resolution of 2.5 Å (3), as the best choice both for receptor and ligand. The amino acid side chains in the final models were positioned using SCWRL (9). Subsequently, the models were refined in YASARA using the Yamber2 force field and the associated protocol (10) until the WHAT IF (11) quality indicators (Ramachandran plot, backbone conformation, and three-dimensional packing quality) converged.

RESULTS

Homology model of NRG1β binding to ErbB3 and ErbB4

Based on the backbone structure of TGFA and ErbB1, as determined by X-ray analysis of their complex, we derived a homology model for the interaction of NRG1β with ErbB3 and ErbB4. Table 1 surveys the potential positive constraints in NRG1β that mediate binding to ErbB3 and ErbB4. In the case of ErbB3, these are located within the N-terminal linear region (H2/L3), A1-loop (K11/F13), A2-loop (V15, N16), B-loop (F21, V23, L26, S27, N28, P29, R31, Y32), C-loop (N38, E39, T41, D43, R44) and C-terminal linear region (Q46, N47, Y48, M50, A51), giving rise to a total of 24 residues that can potentially form a hydrogen bridge, a salt bridge, or a hydrophobic interaction site with residues in this receptor. The interaction sites for binding of NRG1β to ErbB4 were found to be highly similar to those for binding to ErbB3, in agreement with data from Luo et al. (8). An overview of positive and negative constraints is shown in Figure 1. We have previously shown that exchange of the C-terminal linear region of NRG1β for the sequence YYDLL enhances its binding affinity for ErbB3. Homology modeling shows that this C-terminal sequence enhances the total amount of positive constraints with this receptor (Figure 1).

A comparison between the homology structure of NRG1β/ErbB3 and the experimental structure of TGFA/ErbB1 shows that the residues that mediate high affinity binding with their receptor are located at similar positions in both ligand molecules. So, although the primary structure of these positive constraints in EGF and NRG1β are not necessarily conserved, in general the hydrogen bridges, salt bridges and hydrophobic pockets are located on the same positions in the receptor-ligand complex. However, a more close comparison also shows interesting differences. First of all, the B-loop of NRG1β is three amino acids longer than that of EGF, and as a result NRG1β can form two additional hydrogen bounds with the L1 domain of ErbB3

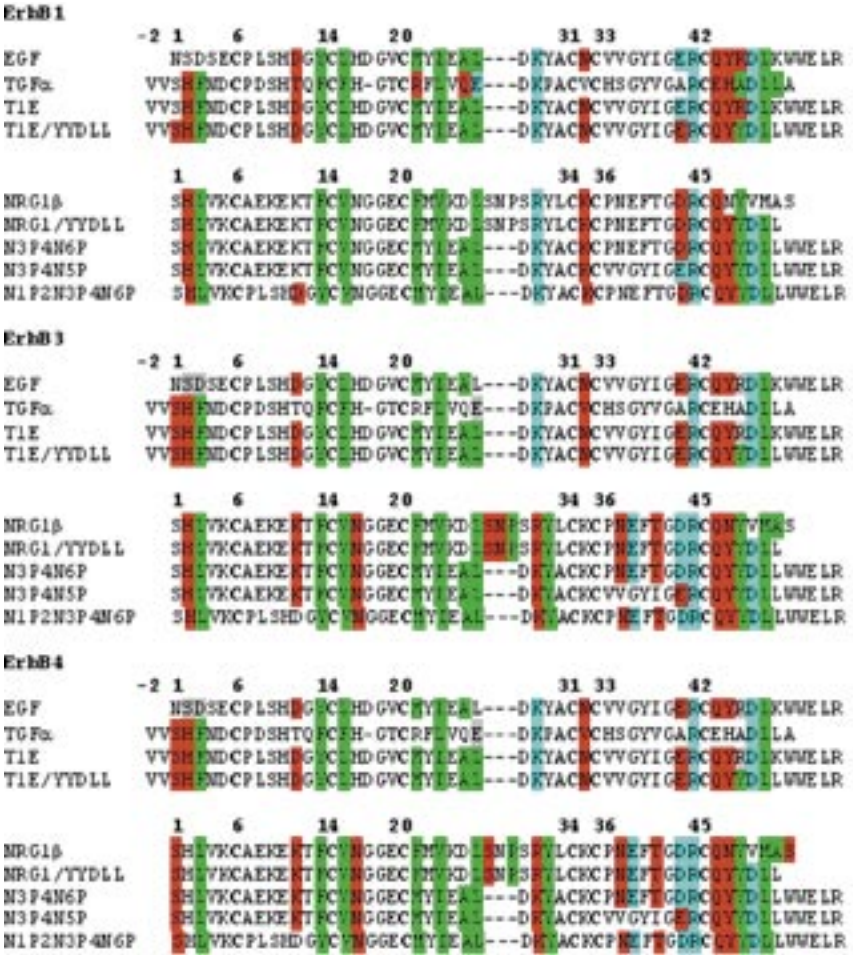

Figure 1. Interaction sites for binding of EGF-like growth factors to ErbB1, ErbB3 and ErbB4, based on bioinformatics homology models. Presented sequences are of human EGF, TGFA , T1E, and NRG1β, as well as T1E/YYDLL (panerbin) and NRG/panerbin chimeras. Conserved cysteines are numbered and depicted in bold. Residues in red are involved in hydrogen bond formation, residues in green in hydrophobic interactions, and residues in blue in salt bridge formation with the indicated ErbB receptors. Negative constraints are depicted in grey, when known. Different ligands may share a residue on the same position in the linear representation, of which one interacts with a receptor and the other does not, because of differences in rotamer positions of the amino acids determined by the surrounding sequences. Indicated interactions of N3P4N6P and N1P2N3P4N6P with ErbB1 are predicted on basis of the homology models of panerbin with ErbB1. Ligands indicated with an asterisk do not show detectable binding affinity for the indicated ErbB receptor. The putative positive constraints of these ligands are counteracted by the presence of negative constraints.

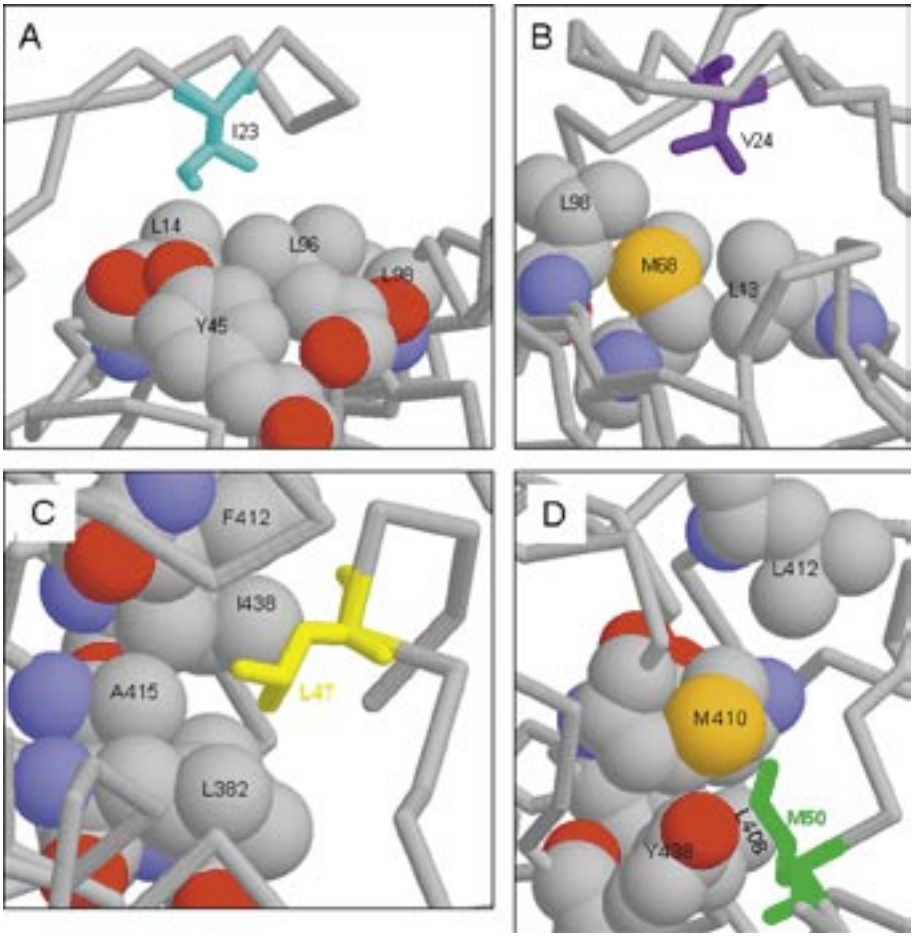

Figure 2. Interaction of B-loop residues with the L1 domain of the ErbB receptors. (A) Interaction of NRG1β with ErbB3. S27 in NRG1β can makes a hydrogen bond to E129 in ErbB3. N28 in NRG1β makes a hydrogen bond with Y125 in ErbB3. (B) Interaction of EGF with ErbB1. Corresponding residues of S27 and N28 in NRG1β and E127 and Y125 in ErbB3 are depicted in similar colors in EGF and ErbB1, respectively. S127 and R125 the corresponding residues of E127 and Y125 in ErbB3 are not involved in hydrogen bond formation with EGF and will also be unable to make hydrogen bonds with NRG1β.

(Figure 1). Interestingly the corresponding residues in ErbB1 would not allow the formation of such additional hydrogen bridges with a ligand containing a 13-amino acids B-loop (Figure 2). Moreover, mutation studies have shown that reducing the length of the B-loop of NRG1β from 13 to 10 residues, thereby deleting this so-called omega loop, does not impair its binding affinity

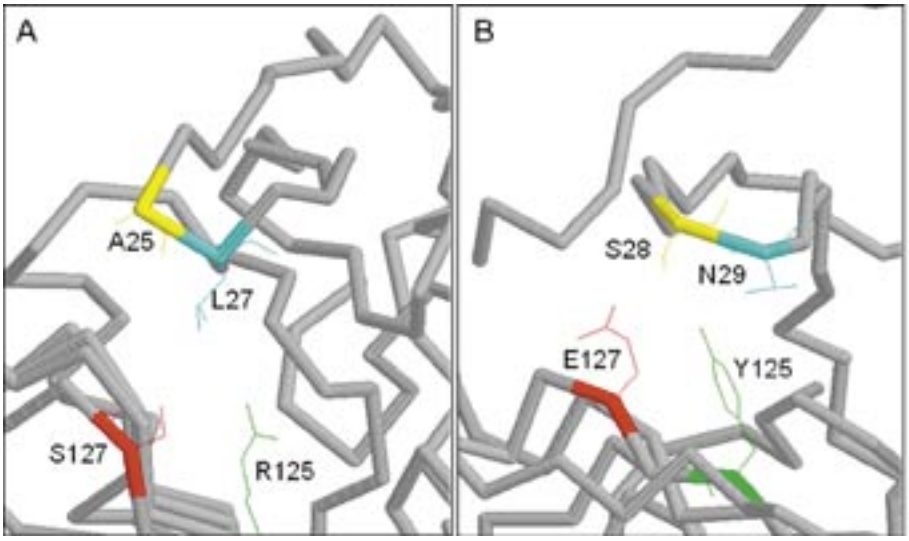

Figure 3. Hydrophobic pockets. (A and C) Ras-Mol representations of a close-up of the homology model of ErbB1 in complex with EGF based on the crystal structure of ErbB1 with TGFA (3). (B and D) Ras-Mol representations of a close-up of the homology model of ErbB3 in complex with NRG1β based on the crystal structure of ErbB1 with TGFA (3). (A) I23 of EGF (cyan) binds to the hydrophobic pocket of the ErbB1 L1 domain. (B) V24 of NRG1β (purple) binds to the hydrophobic pocket of the ErbB3 L1 domain. (C) L47 of EGF (yellow) binds deeply into the hydrophobic pocket of the ErbB1 L2 domain. (D) M50 of NRG1β (yellow) lies on top of the hydrophobic domain of the ErbB3 L2 domain.

for ErbB3 and ErbB4 (12). These data therefore show that the length of the B-loop is not the decisive factor in the receptor specificity of NRG1β.

Secondly, ErbB1 has a relatively deep hydrophobic pocket in each of its two ligand binding domains (L1 and L2) in which the EGF residues I23 (L23 in TGFA) and L47 can penetrate deeply (Figure 3). The corresponding hydrophobic pocket in the L1 domain of in ErbB3 and ErbB4 is less deep, and filled with the smaller hydrophobic residue V24 of NRG1β. Introduction of the I23V mutation in EGF results in a 2-fold lower affinity for ErbB1, indicating that the depth of the hydrophobic pocket in the L1 domain does not explain why NRG1β does not bind ErbB1.

Thirdly, we have shown in a number of studies that sequences in the C-terminal linear region of EGF-like growth factors greatly affect their ErbB affinity and selectivity. Phage display analysis of ligands with a randomized C-terminal liner region have indicated that the residues D46 and L47 in EGF and TGFA are very important for high affinity binding to ErbB1 (5). These two positive constraints are missing in NRG1β, which has a valine and a methionine residue instead. Both the EGF mutant D46V and L47M has a

strongly reduced binding affinity for ErbB1, indicating that the C-terminal linear region of NRG1β does not permit high affinity binding to ErbB1. On the other hand we have shown that a C-terminal linear region consisting of the residues YYDLL is compatible with high affinity binding to all three ligand binding ErbB receptors. Still we observed that the mutant NRG1/YYDLL, which has enhanced affinity for ErbB3, is fully unable to bind ErbB1. These data indicate that the primary sequence of the C-terminal linear region of NRG1β alone does not explain why it is not a ligand for ErbB1.

Exchange of each ErbB3 residue in the NRG1β/ErbB3 homology model for the corresponding ErbB1 residue, while maintaining the overall backbone structure, did not result in a steric clash between the ligand and the receptor molecule. Furthermore, almost all positive constraints between NRG1β and ErbB3 were maintained upon introducing ErbB1 residues. It therefore appears that the inability of NRG1β or NRG1/YYDLL to bind ErbB1 does not result from a lack of positive constraints.

A1-loop and C-loop sequences of NRG1β prevent interaction with ErbB1

In order to identify potential negative constraints in NRG1β for binding to ErbB1, we first introduced the 10-amino acids B-loop of EGF and the generally applicable C-terminal linear region YYDLL into NRG1β. The resulting mutant, designated N3P4N6P (in which P stands for panerbin with sequence T1E/YYDLL), did show high affinity for ErbB4, but fully lacked affinity for ErbB1 (Figure 4A and 4B). Amino acid sequences of the chimeric growth factors and an overview of their positive and negative constraints are shown in Figure 1. We subsequently introduced additional intercysteine domains of panerbin into N3P4N6P, in order to test which regions of NRG1β prevent binding to ErbB1. The rationale behind this approach is that both panerbin and N3P4N6P bind ErbB3 and ErbB4 with high affinity, while only panerbin binds in addition to ErbB1. Mutants obtained by introducing domains from panerbin into N3P4N6P will therefore retain their affinity for ErbB3 and ErbB4, while gaining affinity for ErbB1. Figure 4A shows the binding affinity of the mutant ligands, tested as protein A-fusion proteins, for ErbB4 and Figure 4B for ErbB1. All mutants tested have high affinity for ErbB4, as expected, although for unknown reasons the shape of the displacement curve differs from mutant to mutant. Figure 4B shows that introduction of A1-loop (mutant N1P2N3P4N6P) or C-loop (mutant N3P4N5P) of panerbin into N3P4N6P, results in a gain of affinity for ErbB1, although not to the level of panerbin itself. Protein A-fusion proteins may contain an unknown fraction of misfolded protein, and therefore we purified the active A1-loop mutant (N1P2N3P4N6P) to full homogeneity by cleavage of the protein A–tag and subsequent purification by RP-HPLC. Figure 4C shows that N1P2N3P4N6P has a similarly high affinity for ErbB4 as N3P4N6P, while figure 4D shows that N3P4N6P has no affinity for ErbB1

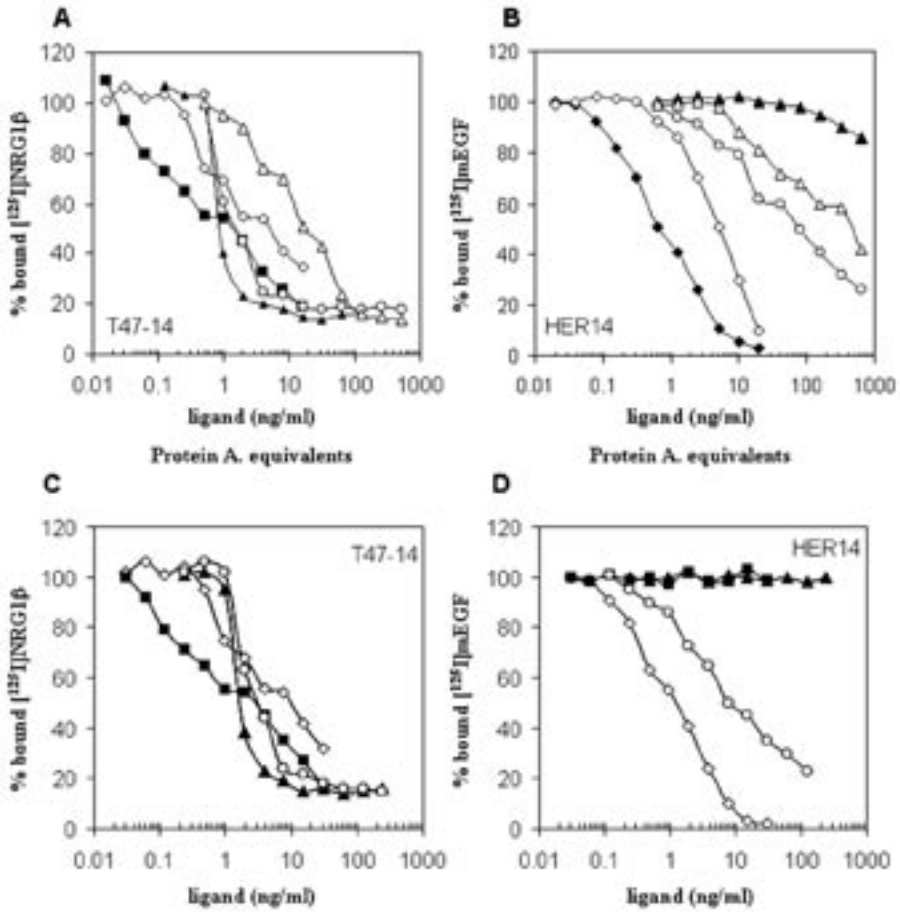

Figure 4 Binding affinities of panerbin-NRG1β chimeras for ErbB1 and ErbB4. (A and C) Ligand binding displacement analysis on ErbB4 overexpressing T47-14 cells treated with 1 ng/ml [^{125}I]-NRG1β. (B and D) Ligand binding displacement analysis on ErbB1 over-expressing HER14 cells treated with 1 ng/ml [^{125}I]-EGF. (A and B) Cells were incubated with increasing amounts of the unlabeled ligands: NRG1β (−■−), Panerbin (T1E/YYDLL) (−◇−), N3P4N6P (−▲−) and N1P2N3P4N6P (−○−). (A and B) Cells were incubated with increasing amounts of Protein A-tagged ligands: protein A-EGF (−◆−), protein A-NRG1β (−■−), protein A-panerbin (T1E/YYDLL) (−◇−), protein A-N3P4N6P (−▲−), protein A-N1P2N3P4N6P (−○−) and protein A-N3P4N5P (−△−). Values of bound radiolabeled ligand in the absence of unlabeled ligand was set on 100%. Results are the mean of at least two independent experiments performed in duplicate.

and N1P2N3P4N6P approximately 10% of the affinity of panerbin. These data indicate that negative constraints in the A1-loop and C-loop of NRG1β impair its interaction with ErbB1.

DISCUSSION

Many studies have been performed on the receptor selectivity of EGF-like growth factors. The requirements for binding of EGF and TGFA to ErbB1 have been studied in particular detail, while we have focussed in previous studies on the question why EGF and TGFA are unable to bind ErbB3 and ErbB4. We have shown both experimentally and by homology modeling that EGF and TGFA can form sufficient interaction sites with ErbB3 and ErbB4 to allow high affinity binding, but that interaction with these receptors is impaired by a number of specific ligand residues, which we have referred to as negative constraints. Relieve of these negative constraints (S2D3 and R45 in EGF; E26 in TGFA) appeared sufficient to give both ligands considerable binding affinity for ErbB3 and ErbB4, while maintaining wild-type affinity for ErbB1. In the present study we have used a similar approach to understand why NRG1β is able to bind ErbB3 and ErbB4, but not ErbB1. Our homology models show that binding of NRG1β to ErbB1 is not impaired by a steric clash between ligand and receptor, while in addition sufficient potential positive constraints appear present to allow considerable binding affinity of NRG1β to ErbB1. Furthermore, by exchanging domains between NRG1β and panerbin, we have shown experimentally that NRG1β contains putative negative constraints for binding to ErbB1 within its A1-loop and C-loop.

The most conspicuous difference between EGF or TGFA and NRG1β is the length of the B-loop. However, it has been established previously that reducing the length of the NRG B-loop from 13 to 10 residues, similarly as in EGF, does not affect its binding affinity for ErbB3 and ErbB4 (12). Moreover, it is known from mutational analysis that the C-terminal linear sequence of NRG1β (NYVMAS) does not facilitate high affinity binding to ErbB1 (1), particularly due to the lack of D46 and L47 as in EGF and TGFA. However,, various studies have shown that this wild-type sequence is also not optimal for binding to ErbB3 (12). Exchange of this sequence to YYDLL, which has been found by us as the optimal C-terminal linear region for binding to all three ligand-binding ErbB receptors, enhances the binding of NRG1β to ErbB3, but does not allow this ligand to bind ErbB1 (12). In fact, our present results show that introduction of the B-loop of EGF, in combination with the YYDLL sequence into the C-terminal linear region (mutant N3P4N6P), is not sufficient to allow any binding of NRG1β to ErbB1.

Upon introduction of further panerbin (or EGF) sequences into N3P4N6P, we derived two mutants with detectable binding affinity for ErbB1. The ligand with the highest binding affinity for ErbB1 was obtained by introducing the A1-loop of EGF into N3P4N6P, giving rise to the mutant

N1P2N3P4N6P. This suggests that the A1-loop of EGF may contain a strong positive constraint or that the A1-loop of NRG1β contains a strong negative constraint for ErbB1. Two amino acids in the EGF A1-loop are directly involved in ErbB1 binding; D11 and Y13. D11 makes a hydrogen bound with S354 of ErbB1, and mutation of D11 into an isoaspartic acid, which will most likely disrupt the hydrogen bond, results in a five–fold loss of affinity (13). The corresponding residue of D11 in NRG1β is K11, but it is currently unclear if K11 forms a negative constraint for ErbB1. Furthermore, Y13 in EGF makes a ring stacking with F355 in ErbB1, which may be shared by F13 in NRG1β. Intriguingly, however, the other residues in the A1-loop are oriented away from the receptor molecule and are therefore not directly involved in receptor-ligand binding.

Introduction of the C-loop of EGF in N3P4N6P also resulted in a mutant (N3P4N5P) with detectable affinity for ErbB1. Two residues of the EGF C-loop are directly involved in ErbB1 interaction; E40 and R41. The corresponding positions in the NRG1β C-loop contain D40 and R41. Both of these residues would be able to make similar interactions with ErbB1. So other residues within the NRG1β C-loop (sequence PNEFT) are most likely responsible for the negative constraint which prevents binding of NRG1β to ErbB1. Again these residues in the C-loop are oriented away from the receptor. More detailed studies will be needed to clarify the role of the A1- and C-loop in the receptor selectivity of NRG1β.

It can be speculated that the A1-loop and C-loop play a, so far not recognized, subtle role in the conformation of the ligand and thereby directly affects receptor-binding affinity. It should be emphasized that the homology models used in this study have been based on the backbone structure of TGFA in complex with ErbB1, and not on the solution structure NRG1α. These structures are highly similar, but subtle differences cannot be excluded.

It appears that the lack of binding affinity of NRG1β for ErbB1 is not due to the presence of a limited number of residues that act as negative constraint, as observed for the lack of binding of EGF and TGFA to ErbB3. Our data indicate that for reasons not well understood each of the intercysteine domains contributes to a certain extent to the lack of binding affinity of NRG1β to ErbB1. The more domains from panerbin are introduced into NRG1β, the higher the affinity for ErbB1, but so far it has not been possible to pinpoint a few residues that due to steric hindrance or electrostatic interaction impair NRG binding to ErbB1.

Exchange of single amino acids between the EGF and NRG1β A1-loop or C-loop will identify those residues in the NRG1β A1-loop and C-loop that prevent ErbB1 binding. Identification of negative constraints in NRG1β for ErbB1 and subsequently the nature of this negative constraint (steric clash, charge repulsion or subtle change of conformation) will contribute to our understanding of how receptor selectivity of ErbB ligands is regulated.

Table 1.

Residue in EGF	Function in ErbB1 binding	Residue in TGFα	Function in ErbB1 binding	Residue in NRG1β	Function in ErbB3 binding	Function in ErbB4 binding
		V-2	NDC			
		V-1	NDC	T1	NDC	NDC
N1	Disordered %	S1	NDC	S2	NDC	NDC
S2	Disordered %	H2	HB: Y101	H3	HB: T102	NDC
D3	Disordered %	F3	HF: L14, L69 and Y45	L4	HF: L13, M68, M97 and L98	HF: L13, L68 and L98
S4	Disordered %	N4	NDC	V5	NDC	NDC
E5	NDC	D5	NDC	K6	NDC	NDC
C6	NDC	C6	NDC	C7	NDC	NDC
P7	NDC	A7	NDC	A8	NDC	NDC
L8	NDC	D8	NDC	E9	NDC	NDC
S9	NDC	S9	NDC	K10	NDC	NDC
H10	NDC	H10	NDC	E11	NDC	NDC
D11	HB: S353	T11	HB: S353	K12	HB: N346	HB: 351
G12	NDC	Q12	HB: R353	T13	NDC	NDC
Y13	RS: F357	F13	RS: F357	F14	RS: W350	RS: Y355
C14	NDC	C14	NDC	C15	NDC	NDC
L15	HF: V350	F15	HF: V350	V16	HF: T343 and I342	HF: T348 and V347
H16	NDC	H16	NDC	N17	HB: K411	HB: Q416
D17	NDC			G18	NDC	NDC
G18	NDC	G18	NDC	G19	NDC	NDC
V19	NDC	T19	NDC	E20	NDC	NDC
C20	NDC	C20	NDC	C21	NDC	NDC
M21	HF: L14, L69 and Y45	R21	C-beta's HF: L14, L69 and Y45, HB: Q16	F22	HF: L13, M68, M90 and L98	HF: L13, L68 and L98
Y22	NDC	F22	NDC	M23	NDC	NDC
I23	HF: L14, L69 and Y45	L23	HF: L14, L69 and Y45	V24	HF: site 1 L13, M68, M97 and L98	HF: L13, L68 and L98

				L27	HF L98	HF: L98
				S28	HB: E127	HB: E127 and Q128
				N29	HB: Y125	ND C
L26	HF: L14, L69 and Y45	E26	SB: R125 C-beta's HF: L14, L69 and Y45	P30	HF: L13, M68, M97 and L98	HF: L13, L68 and L98
D27	ND C	D27	ND C	S31	ND C	ND C
K28	SB: E90	K28	SB: E90	P32	C-beta's HF: L13, M68, M90 and L98, SB: E89	C-beta's HF: L13, L68 and L98
Y29	ND C	P29	HF: F25	Y33	ND C	ND C
A30	ND C	A30	ND C	L34	HF: L13, M68, M97 and L98	HF: L13, L68 and L98
C31	ND C	C31	ND C	C35	ND C	ND C
N32	HB: Q18	V32	ND C	K36	ND C	ND C
C33	ND C	C33	ND C	C37	ND C	ND C
V34	ND C	H34	ND C	P38	ND C	ND C
V35	ND C	S35	ND C	N39	HB: N21	HB: Q21
G36	ND C	G36	ND C	E40	SB: K411	ND C
Y37	ND C	Y37	ND C	F41	ND C	ND C
I38	ND C	V38	ND C	T42	HB: N11	HB N11
G39	ND C	G39	ND C	G43	ND C	ND C
E40	SB: K13	A40	ND C	D44	ND C	ND C
R41	SB: D355	R41	SB: D355	R45	SB: D348	SB: D353
C42	ND C	C42	ND C	C46	ND C	ND C
Q43	C-beta's HF: F357 and V350 *	E43	C-beta's HF: F357 and V350 *	Q47	HB: T348	HB: T348, Q323
Y44	HB: H346	H44	HB: Q408	N48	HB Y401	HB N380
R45	HB: S416	A45	HF: site 3	Y49	HB: S55	HB: D439
D46	SB: R29 N	D46	SB: R29 N	V50	ND C $	ND C $
L47	HF: L382, F412, A415 and	L47	HF: L382, F412, A415 and I438	M51	HF: L408, M410, L412,	HF: L408, M410, L412,
K48	HB Q411	L48	HF: site 3	A52	HF: L408, M410, L412,	HF: L408, M410, L412,
W49	ND C	A49	ND C	S53	HB: Y51	HB: Y436

Table 1. Interactions of EGF with ErbB1 and NRG1β with ErbB3 and ErbB4. The amino acids in EGF and TGFA that are directly involved in ErbB1 binding were deduced from the crystal structure of EGF or TGFA in complex with ErbB1. The amino acids of NRG1β that are directly involved in receptor binding were identified by homology modeling of NRG1β in complex with ErbB3 and ErbB4 based on the crystal structure of TGFA in complex with ErbB1. % Disordered structure is based on NMR data (13). # D46 makes a intern hydrogen bond tot the backbone of K48 thereby bending the backbone and pushing L47 deep into the hydrophobic pocket at interaction site 3. $ Backbone bending not needed since hydrophobic domain at site 3 in ErbB3 is less deep. HF: hydrophobic interaction, HB: hydrogen bound, SB: salt bridge. NDC: no direct contact with the receptor.

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8

Humanization of the Drosophila epidermal growth factor receptor ligand Spitz bridges the Drosophila and human ErbB networks

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ABSTRACT

The EGF receptor signaling network in *Drosophila melanogaster* consists of a single receptor (DER), which can be activated by four distinct ligands, designated Spitz, Keren Gurken and Vein. The overall structure of these ligands is very similar to that of human EGF, but so far there is no indication that human and Drosophila EGF-like growth factors are able to bind to each other's receptors. In this study we have made the four Drosophila EGF-like growth factors as recombinant proteins and have shown that none of them is able to bind human ErbB1. Furthermore, we showed that human EGF cannot bind DER. In order to understand the receptor specificity of EGF and Spitz, we made chimeras by exchanging intercysteine domains between the two proteins. Our data show that EGF in which the region between the third and the fifth cysteine has been replaced by Spitz sequences, binds with low affinity to both ErbB1 and DER, and is able to induce MAPK activation in both DER overexpressing S2DER cells and ErbB1 overexpressing HER14 cells. Our results indicate that the receptor specificity of Spitz and DER does not result from structural differences between these ligands and their receptors, but more from evolutionary differences in individual amino acids that impair binding to each other's receptors.

INTRODUCTION

The ErbB signaling network is highly conserved among multicellular organisms and plays an important role in growth and development. During evolution the ErbB signaling network has evolved from a relatively simple receptor-ligand complex to a complicated multi-component system. Invertebrates such as *Caenorhabditis elegans* contain one EGF receptor gene (let-23) which is activated by a single ligand (lin-3) (1). The fruitfly *Drosophila melanogaster* (2) also contains a single EGF receptor gene (DER), but this receptor is regulated by four distinct EGF-like growth factors, designated Spitz, Keren, Gurken, and Vein (3). In urochordates two distinct receptor genes have been identified, most likely as a result of a first whole genome duplication (Van Leeuwen et al., unpublished). A second whole genome duplication has resulted in the four ErbB receptors present in mammals (ErbB1-4), which can be activated by a total of eleven distinct ligands. ErbB1 can be activated by a.o. EGF and TGFA and is expressed in almost all tissues. Studies with knockout mice have shown that ErbB1 functions in the development of lung, epidermis, placenta and mammary gland (4-6). Furthermore, ErbB1 is overexpressed in many, particularly epithelial tumors, and is therefore an important target for anti-tumor drugs.

The *Drosophila* EGF receptor DER plays an important role in eye and

wing development of the fly. The key regulatory step for activation of this receptor is the proteolytic processing of membrane-bound Spitz, Keren and Gurken by proteolytic enzymes, including Star and Rhomboid. Spitz and Keren are ubiquitously expressed during *Drosophila* development, while Gurken is only transcribed in germline cells and activates DER in the follicle cells of the ovary. Finally, Vein is a secreted ligand with weaker activation capacity and is involved in muscle attachment. Furthermore, the *Drosophila* EGF receptor system is unique in that it contains in addition a secreted antagonist, named Argos. This antagonist has an EGF-like structure and prevents DER binding of a.o. Spitz. (7)

The extracellular domain of ErbB receptors contains two leucine-rich domains (L1 and L2) that are involved in ligand binding and are spaced by two cysteine-rich domain (C1 and C2), followed by a transmembrane region and an intracellular tyrosine kinase domain. Upon ligand binding ErbB receptors undergo a conformational change from an auto-inhibited to an open state, and consequently the C1 domain becomes available for receptor dimerization (8). DER resembles the mammalian family members in its overall structure but for unknown reasons its second cysteine-rich domain is extended as a result of duplication (9).

All EGF-like growth factors share an overall structure of approximately 50 amino acids with a conserved spacing of cysteines that form three bridges and thereby give rise to three looped regions, designated A-, B- and C-loop. From crystal structure analysis of the EGF/ErbB1 and the TGFA/ErbB1 complex, it is known that EGF-like growth factors contain approximately 20 residues that directly interact with their receptor (10, 11). These residues, which we have referred to as positive constraints, form the basis for the observed high affinity receptor interaction. Spitz and human EGF show 32% and the ligand binding domains of DER and ErbB1 more than 40% sequence homology. Although the primary structure of Spitz resembles its mammalian orthologues to a large extent, Spitz does not appear to bind human ErbB receptors. This might be due to the fact that it is unable to fulfill sufficient positive constraints with mammalian ErbB receptors for high affinity interaction, or because it contains residues that by steric hindrance or charge repulsion prevent binding to these receptors.

In present study we have investigated the compatibility between the human ErbB system and the DER system. Therefore we stimulated DER overexpressing S2DER cells with several ErbB ligands and stimulated ErbB1 overexpressing cells with recombinant Spitz, Keren, Vein and Gurken. To determine why human EGF does not bind and activate DER and why *Drosophila* Spitz does not bind and activate human ErbB1, we designed several hEGF-Spitz chimeras. Based on these functional studies in combination with homology models of the Spitz-DER complex, we have identified a number of amino acids in Spitz that impair its binding to ErbB1.

EXPERIMENTAL PROCEDURES

Cell lines

Interleukin 3-dependent murine 32D hematopoietic progenitor cells transfected with distinct human ErbB-encoding plasmids were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS, GibcoBRL Life Technologies) and 0.25 ng/ml murine interleukin 3 (mIL-3, Promega), and kept subsequently under continuous selection by addition of 0.6 mg/ml G418 (Calbiochem). ErbB1 overexpressing HER-14 fibroblasts (*12*) and ErbB4 overexpressing T47-14 fibroblasts, both derived from the parental murine NIH3T3 cell line, were cultured in gelatinized flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS).

Production of EGF-Spitz chimeras

DNA encoding Spitz-EGF chimeras were constructed using splice overlap extension PCR (*13*), using human EGF and *Drosophila* Spitz cDNA (kindly provided by Dr. Matthew Freeman, MRC-Laboratory of Molecular Biology, Cambridge, UK) as templates. The 5'-primer contained a linker (sequence: GGATCCATTGAAGGTCGT) with a BamH1 restriction site as well as a sequence encoding the amino acid sequence IEGR, that can be cleaved by the Factor X endoprotease, while the 3'-primer (sequence: GTCGACTTA) contained a stop codon and a SalI restriction site. The inserts were introduced into the pEZZ vector using the BamHI and SalI sites, and the obtained constructs verified by automated cycle sequencing. Recombinant ligands were expressed as a protein A-tagged fusion proteins in the protease K-deficient *E.coli* strain KS474. Chimeras were purified by affinity chromatography using an IgG-sepharose column followed by RP-HPLC. The amount of protein A-tagged Spitz-EGF chimera was calculated from the peak area (absorption at 229 nm) in the RP-HPLC chromatogram, using murine (m)EGF as a standard.

Production, purification and quantification of myc-tagged DER ligands

Since we did not succeed in producing Vein and Gurken in *E.coli* we used the *Pichia pastoris* protein expression system (Invitrogen) in order to produce all four DER agonists. Thereto, cDNA of the DER ligands (all kindly provided by Dr. Ben-Zion Shilo, Department of Molecular Genetics, Weizmann Institute, Israel) was cloned into the *P. pastoris* expression vector pPICZ_A. (Spitz aa F71-K129, Keren aa F57-T113, Gurken aa I178-Y234 and Vein aa A561-V610) The primers used for PCR introduced a 5 prime *Xho*I and a 3

prime *Xba*I restriction site permitting directional cloning of the amplified DNA in frame with the α-factor signal sequence in the pPICZ_A expression vector. Recombinant DER ligands were expressed in *Pichia pastoris* with an N-terminal cleavable Myc-His-Genenase-1 tag, as described previously (14). In brief, expression of recombinant DER ligands was induced by growing a selected Mut^+ clone on BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 0.04% biotin and 0.5% v/v methanol) for four days at 30°C with additional supplies of methanol every day. DER ligands were purified using Ni-NTA beads (Invitrogen) and dialyzed against 0.5 M HAc, using a dialysis bag with a cut off smaller than 3.5 kDa. The concentration of Myc-tagged recombinant growth factor present in the dialyzed Ni-NTA eluate was quantified by spotting serial dilutions on a nitrocellulose membrane followed by detection with anti-Myc antibodies. Staining was compared with that of a serial dilution of a known amount of Myc peptide (R&D systems).

Receptor phosphorylation and MAPK activation

DER expression was induced in S2DER cells by treating these cells overnight with 0.6 μM $CuSO_4$ in Schneider's medium supplemented with 0.5% FCS . A total of $1.0x10^6$ S2DER cells were subsequently stimulated for 5 min at 30°C with 20 ng recombinant DER ligand in a 100 μl cell suspension. Cells were then spun down and washed with ice-cold PBS after which they were rapidly lysed in 100 μl of lysis buffer supplemented with protease inhibitors (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 15 mM Na-deoxycholate, 0.1% SDS, 1.5 mM $MgCl_2$, 1 mM PMSF, 5 μg/ml pepstatin A, 0.15 units/ml aprotein, 5 μg/ml leupeptin, 2mM Na_3VO_4). Lysates were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using 8% acrylamide gels, followed by a 1 hour transfer at 300 μA to a nitrocellulose membrane, wetted in Tween containing Tris-buffered saline (TBST: 10 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween-20) with 5% BSA. Subsequently the membranes were cut horizontally in two pieces at a height corresponding to the 100 kDa region, after which the upper part was incubated overnight at 4°C with a 1:2.000 dilution of PY20 anti-phosphotyrosine antibodies (Upstate Biotechnology), while the lower part was incubated for 1 hour at 4°C with a 1:2.000 dilution of anti-phosphorylated MAPK antibodies (Cell Signaling) in TBST/BSA. Both incubations were followed by a 1 hour treatment with a 1:10.000 dilution of peroxidase-conjugated rabbit-anti-goat antibodies (Cell Signaling) in TBST/BSA. After each incubation step the membranes were washed in TBST with constant agitation. Blots were visualized using enhanced chemoluminiscence (ECL). Dual stain molecular weight markers (range 10-250 kDa) were obtained form BioRad.

Radioligand displacement analysis

Natural mEGF (Bioproducts for Science) and recombinant protein A-tagged Spitz were iodinated enzymatically to a specific activity of 1.1 Ci/μmol (15). Ligand binding displacement studies on HER14 were performed as described previously (16). Ligand binding displacement studies on S2DER cells were performed in suspension. To $2.5x10^6$ $CuSO_4$- treated S2DER cells in 0.1 ml Schneider's Medium/10% BSA, 0.1 ng of [^{125}I]-protein A-tagged Spitz was added in the additional presence of serial dilutions of unlabeled ligand. After incubation for 2 hours at room temperature, cells were spun for 1 min in a microcentrifuge and washed to remove unbound [^{125}I]-protein A-tagged Spitz. Cells were then resuspended in 0.5 ml of Schneider's medium with 10% FCS and 2% BSA, loaded onto a cushion of 0.7 ml undiluted FCS and spun down in a microcentrifuge for 1 min at 13.000 rpm. Radioactivity in the cell pellets was measured by γ-counting.

RESULTS

DER ligands form high molecular weight complexes in Pichia pastoris

We have previously shown that mammalian EGF-like growth factors can be functionally expressed in *Pichia pastoris*. In order to obtain recombinant DER ligands we used a similar approach after linking their genes to a cleavable Genenase I-Myc-His tag. Figure 1A shows that after isolation on Ni-NTA beads from the culture supernatant, the various DER ligands showed high molecular weight complexes with apparent sizes of 25-75 kDa, far exceeding the expected size of approximately 8.5 kDa. In contrast to mammalian EGF-like growth factors, Spitz, Gurken and Vein contain potential glycosylation signals in their EGF-like domain and since *Pichia pastoris* is known for its extensive mannose glycosylation, we determined whether the obtained recombinant ligands were glycosylated. Figure 1B and 1C show that cleavage of the sugar chains by endoglycosydase F treatment resulted in reduced molecular sizes of 15-25 kDa for Spitz, Gurken and Vein, while the molecular size of Keren, which lacks a glycosylation site, was unaffected by endoglycosidase F treatment. Furthermore it appeared that the obtained ligands showed intramolecular disulphide bridges, since their apparent molecular sizes were significantly reduced when the SDS gels were run under reducing conditions (Figure 1B). These data show that recombinant expression of DER ligands in *Pichia pastoris* is far more complex than previously observed for mammalian EGF-like growth factors.

In order to quantify the amount of Myc-tagged recombinant DER ligands, serial dilutions of Ni-NTA elute were spotted onto blotting paper, together with a known amount of Myc peptide, and stained with anti-Myc antibodies. Figure 1D shows the chemiluminescence intensity of DER ligands

and Figure 1E the conversion of microliters culture medium to nanomoles of Myc-tagged DER ligand. In this way we determined a concentration of 2 nM Spitz, 6 nM Keren, 15 nM Vein and 15 nM Gurken in the NI-NTA elutes.

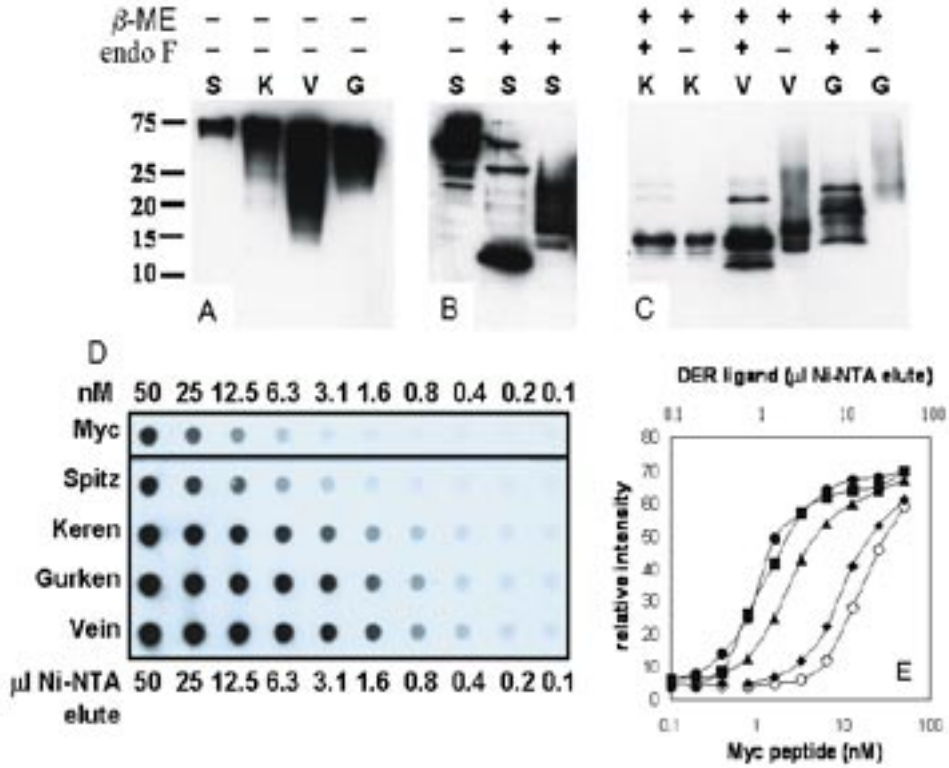


Figure 1. Expression of DER ligands in *Pichia pastoris*. A. Western blot of Myc-tagged recombinant DER ligands under non-reducing conditions. Molecular sizes are indicated in kDa. B. Effect of beta-mercapto-enthanol (β-ME) and endoglycosidase F treatment on the electrophoretic behaviour of Spitz (**S**). C. Effect of β-ME and endo F on the electrophoretic behaviour of Keren (**K**), Vein (**V**) and Gurken (**G**). D. Quantification of Myc-tagged recombinant DER ligands after Ni-NTA purification and spot-blotting of the Ni-NTA elutes as detected by anti-Myc antibodies. Calibration was carried out by spotting the indicated amounts of Myc-peptide E. Quantification of DER ligands based on dose-dependent Myc staining intensity as measured by densitometry. Levels of 50% Myc-tag staining (IC50 value) were estimated graphically from the highest and lowest values obtained experimentally. The following concentrations of the myc-tagged DER ligands were deduced from the IC50 values: Myc peptide (—○—), Gurken (—●—) 20 nM, Spitz (—♦—) 2 nM, Vein (—■—) 20 nM, Keren (—▲—) 6 nM.

Detection of the relative binding affinity of Spitz, Keren, Gurken and Vein for DER and ErbB receptors

To determine the relative affinity of these *Drosophila* ligands for DER, we determined their ability to compete with radiolabeled protein A-tagged Spitz for binding to DER overexpressing S2DER cells. Figure 2A shows that based on Myc-equivalents Spitz (IC50 = 8 nM) has the highest binding affinity for DER, followed by Keren (IC50 = 30 nM) and Gurken (IC50 = 70 nM). At the concentrations tested no binding competition was observed for Vein. Figure 2B shows that Spitz strongly induces DER phosphorylation and MAPK phosphorylation in S2DER cells, while Keren, Gurken and Vein showed only weak effects. Interestingly, also Vein was active in these assays, in spite of the fact that its binding affinity was below detection.

In order to determine whether the DER ligands are able to activate the various human ErbB receptors, HER14 cells (ErbB1), D23 cells (ErbB2 and ErbB3) and T47-14 cells (ErbB4) were stimulated with Spitz, Keren, Gurken and Vein but, as shown in Figure 3, none of the DER ligands was able to induce phosphorylation of one of these ErbB receptors. Furthermore the data

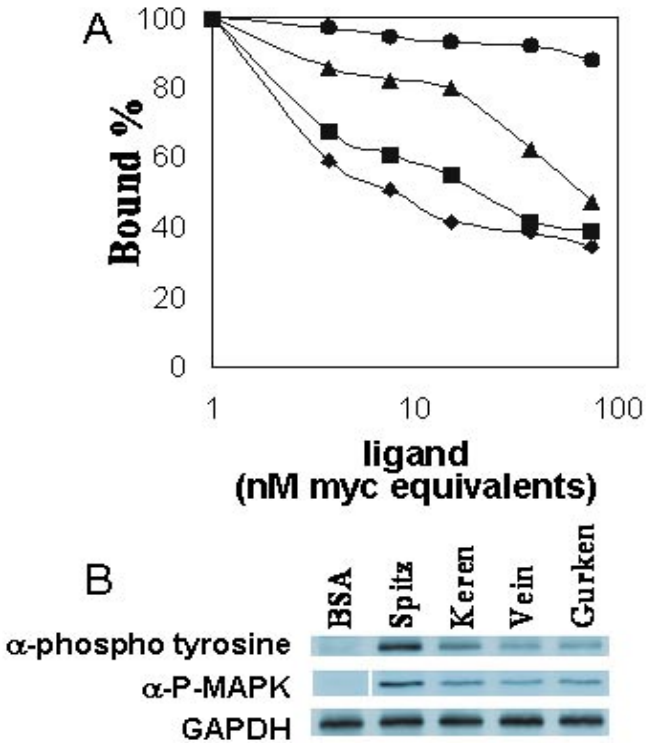


Figure 2. Determination of the binding affinity of Spitz, Keren, Gurken and Vein for DER. A. [^{125}I]-protein A-Spitz binding competition assays on S2DER cells. Vein (—●—), Gurken (—▲—), Keren (—■—), Spitz (—♦—). B. Ligand-induced tyrosine phosphorylation and MAPK phosphorylation. S2DER cells were stimulated with 20 nM ligand for 10 min. BSA was used as negative control. GAPDH was tested as a loading control.

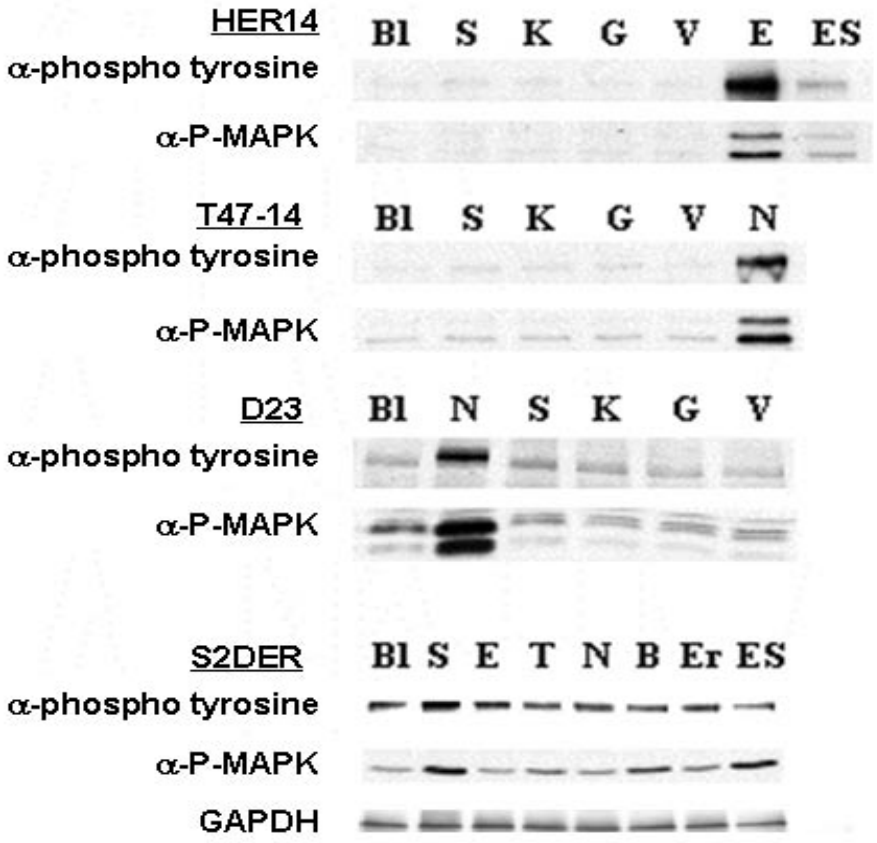


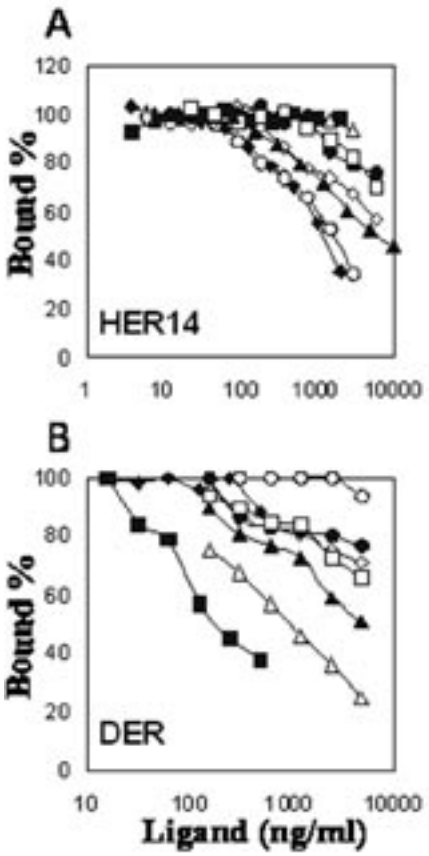
Figure 3. Activation of DER and ErbB1 by mammalian and *Drosophila* EGF-like growth factors, as measured by induced tyrosine phosphorylation and phosphorylation of MAPK. HER14, T47-14, D23 and S2DER cells were stimulated with 20 nM Spitz (S), 20 nM Keren (K), 20 nM Gurken (G), 20 nM Vein (V), 100 ng/ml EGF (E), 100 ng/ml TGFA (T), 100 ng/ml NRG1β (N), 100 ng/ml BTC (B), 100 ng/ml EREG (Er) and 100 ng/ml of the chimera E3S5E (ES) for 10 min. BSA (Bl) was used as a negative control.

show that none of the mammalian ErbB ligands appeared able to activate DER in a phospho-tyrosine or MAPK assay.

Identification of EGF and Spitz domains involved in binding specificity for DER and ErbB1

In order to identify experimentally which regions in EGF and Spitz are discriminative for receptor binding, we made a set of chimeras between EGF and Spitz, and tested their affinity for both ErbB1 and DER. Chimeras

were made by exchanging domains bordered by the third and fifth cysteine of both ligands. Figure 4A shows that in EGF the sequences N-terminal of the third cysteine can be replaced for the corresponding sequences of Spitz (S3E) without loss of binding affinity for ErbB1. However, exchange of the B-loop and hinge region (E3S5E) or of the C-loop in combination with the linear C-



C

relative binding affinity	hEGFR	DER
EGF	100%	NMA
Spitz	NMA	100%
S3E	80%	NMA
E3S	NMA	15%
S5E	NMA	NMA
E5S	NMA	NMA
S3E5E	NMA	NMA
E3S5E	15%	2%

Figure 4. Determination of the binding affinity of Spitz-EGF chimeras for DER and ErbB1. A. [^{125}I]-mEGF binding competition assay on HER14 cells. B [^{125}I]-protein A-Spitz binding competition assays on S2DER cells. Unlabeled EGF (—♦—), Spitz (—■—), E3S (—Δ—), S3E (—○—), E5S (—◊—), S5E (—●—), E3S5E (—▲—) and S3E5S (—□—) were added at the indicated concentrations. Results are presented as mean of at least three independent experiments performed in duplicate. C. Relative affinities of the EGF-Spitz chimeras for DER and ErbB1, based on the IC50. Data are expressed relative to EGF (for ErbB1) and Spitz (for DER). Ligands that did not reach 50% binding competition within the concentration range tested are indicated as NMA (no measurable affinity).

terminal region (E5S) by Spitz sequences reduced the affinity of EGF for ErbB1 to 10-15%. Figure 4B shows that introduction of EGF sequences N-terminal of the third cysteine of Spitz (E3S) reduced the affinity of the latter for DER to 15%, while EGF with only the B-loop and hinge region of Spitz (E3S5E) showed only 2% of the affinity of Spitz for DER. None of the chimeras tested showed high affinity binding to both DER and ErbB1. Nevertheless, E3S5E showed low but detectable binding affinity for both DER and ErbB1, and was the only ligand that was able to induce ErbB1 phosphorylation and MAPK phosphorylation in both HER14 cells and S2DER cells, as shown in Figure 3.

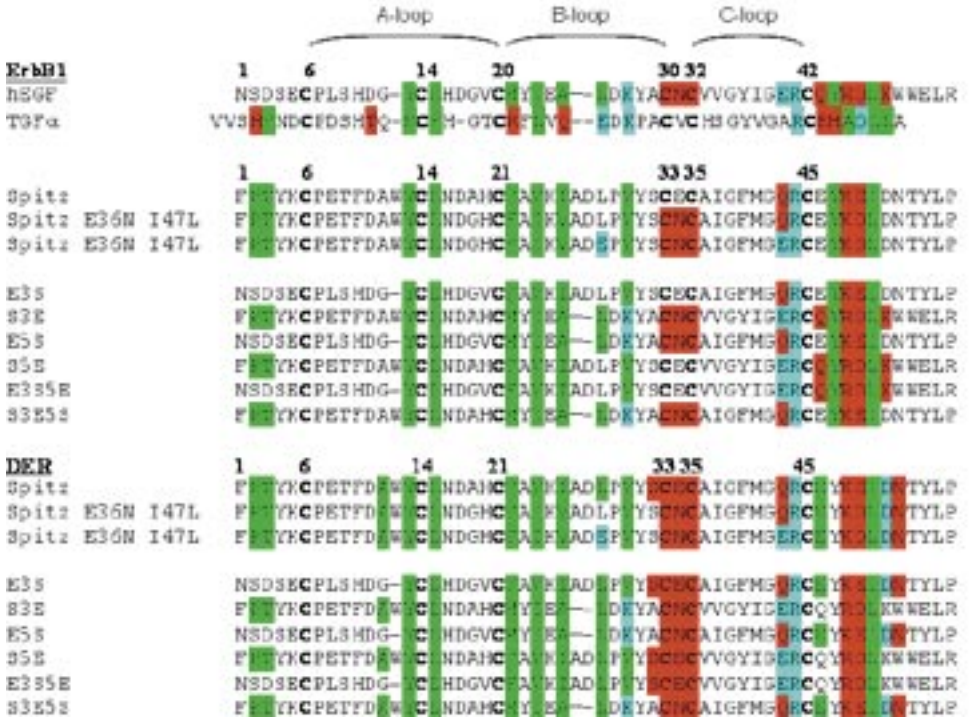


Figure 5. Interaction sites of EGF-Spitz chimeras with ErbB1 and DER, based on homology models. Amino acid numbering is indicated for EGF and Spitz. Colored residues represent the direct interaction sites between the indicated ligand and receptor, as predicted by homology models based on the crystal structure of the TGFA-ErbB1 complex. Residues marked in red in the ligand sequences are involved in hydrogen bond formation, residues in green are involved in hydrophobic interactions, and residues in blue are involved in salt bridge formation with the indicated receptors.

It can be concluded that binding to ErbB1 and DER is not mutually exclusive, but that with the current approach no high affinity ligands were obtained for both receptors.

E35N and I50L mutation is not sufficient for Spitz interaction with ErbB1

In order to understand at the molecular level why Spitz is unable to bind ErbB1 and EGF to bind DER, we used a bioinformatics approach in which the Spitz/DER complex was modeled and subsequently compared to that of EGF/ErbB1. For homology modeling we have made use of the experimental crystal structure of the TGFA-ErbB1 complex *(*10). We have previously shown that the high affinity interaction between EGF and ErbB1 is mediated by three salt bridges, eight hydrogen bonds and eight hydrophobic interactions. A similar analysis on the Spitz/DER complex showed that Spitz can form two salt bridges, eight hydrogen bonds and eleven hydrophobic bonds with DER (Figure 5). Based on amino acid sequence homology between EGF and Spitz we assume that a large number of salt bridges and hydrogen bonds cannot be formed between Spitz and ErbB1, particularly in the hinge region and in the C-terminal linear region. However, no putative charge repulsions or steric clashes were predicted which could impair the binding of Spitz to ErbB1.

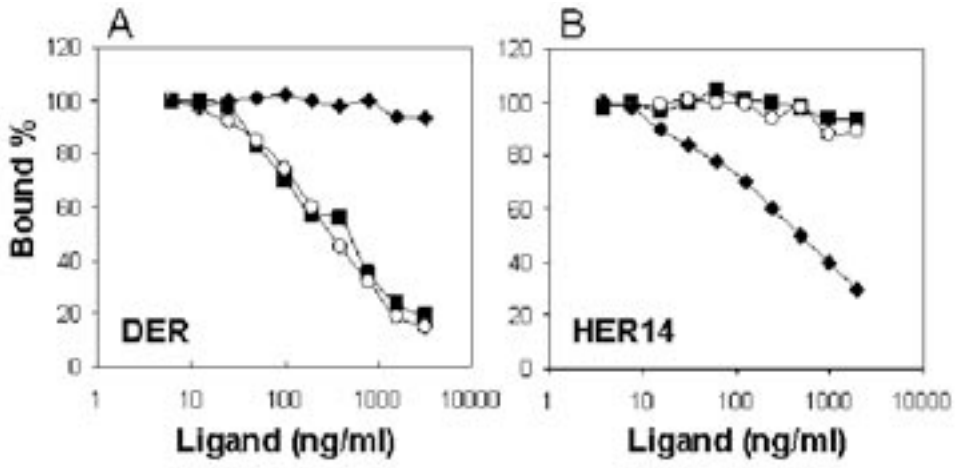


Figure 6. Determination of the ErbB1 binding affinity of Spitz/E33N/I49L. A. [^{125}I]-protein A-Spitz binding competition assays on S2DER cells. B [^{125}I]-mEGF binding competition assay on HER14 cells. EGF (—♦—), Spitz (—■—) and Spitz E35N I50L (—○—). Results are presented as mean of three independent experiments performed in duplicate.

Compared to EGF, Spitz contains an Asp instead of N33 in the hinge region, which in EGF makes a hydrogen bond with Q18 in ErbB1, and an Ile instead of L47 in the C-terminal linear region, which is known to be essential for high affinity binding of ErbB1. Single amino acid mutations in EGF have shown that the mutation N33D results in a four-fold decrease (17) and the mutation L47I in a six-fold decrease (18) in ErbB1 affinity. We therefore tested the mutant Spitz/E35N/I50L for binding affinity to both ErbB1 and

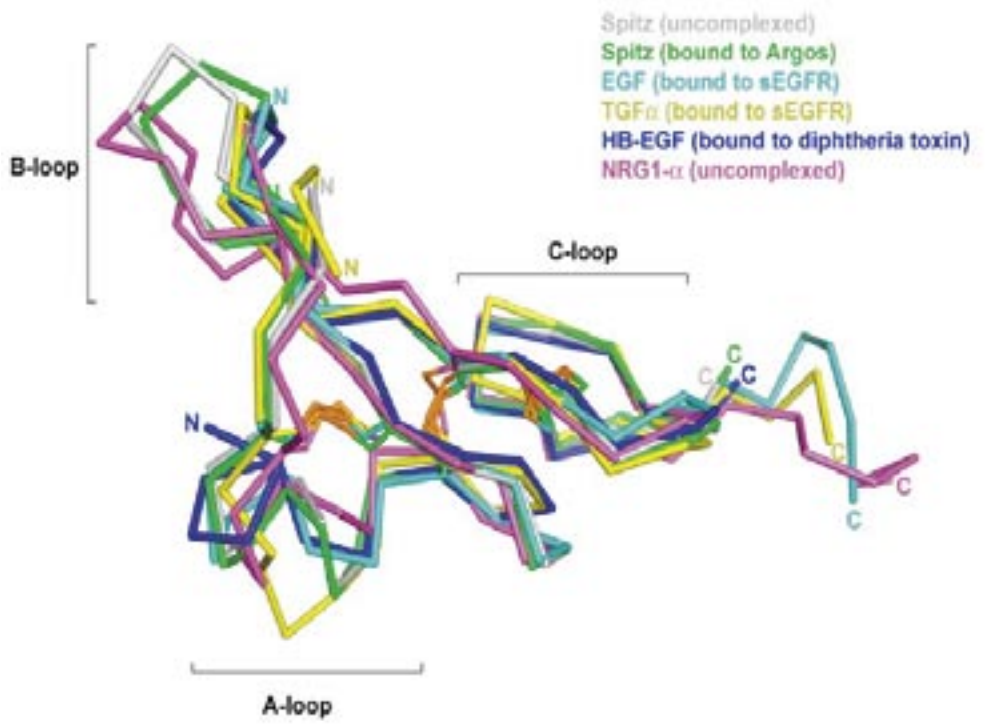


Figure 7. Overlay of EGF domain structures. Unbound (grey) and Argos-bound (green) Spitz are shown overlaid with crystallographically derived (sEGFR-bound) structures of EGF (cyan) and TGFA (yellow), diphtheria toxin-bound HB-EGF (blue/purple), and a NMR structure of free NRG1α (28) (magenta). Note that Spitz most closely resembles NRG1α in the length and approximate conformation at the end of the B-loop. The tip of the B-loop is also the only region in which clear differences between free (grey) and bound (green) Spitz can be discerned. Figure adapted from Klein et al. (24), including data for EGF (11), TGFA (10), HB-EGF (27) and NRG1α (28).

DER. Figure 6 shows that introduction of these two mutations in Spitz did not affect its binding affinity to DER, but the double mutant did not show any detectable binding affinity to ErbB1. It can therefore be concluded that the E35N and I50L mutations in Spitz are not sufficient to make it a high affinity ligand for both ErbB1 and DER.

DISCUSSION

In this study we have shown that recombinant *Drosophila* EGF-like growth factors are able to activate DER but cannot bind to human ErbB receptors. Similarly, all human ErbB ligands tested, in particular EGF, TGFA, NRG, BTC and EREG, were unable to activate DER. Domain-exchange studies between EGF and Spitz resulted in a chimera E3S5E with detectable, but still low binding affinity to both DER and ErbB1.

Binding competition analysis on S2DER cells showed that Spitz is the *Drosophila* ligand with the highest binding affinity for DER, while Vein is only a low affinity binder. This is consistent with literature data in which is has been shown that Spitz is the main DER activation ligand *in vivo*, while Vein as a weak activator is used in tissues where only low levels of DER activation are required (19). None of the DER ligands was able to activate any of the human ErbB receptors in the concentration range tested. In a recent study we have shown that the positively charged R45 in the TGFA-EGF chimera T1E is a negative constraint for binding to both ErbB3 and ErbB4 (20). Furthermore, Hobbs et al. (21) have shown that K48 in NRG2α prevents interaction with ErbB3 while the K48F mutation in NRG2α results in a gain of function for binding to ErbB3. In chapter 4 we showed that R45 in EGF causes a charge repulsions with K415 in ErbB3 and with K418 in ErbB4. Therefore we assume that K48 in Spitz, the corresponding residue of R45 in EGF, will prevent binding to ErbB3 or ErbB4. Remarkably, DER contains a Lys on the corresponding position of K415 of ErbB3, while a Lys on position 48 is conserved in all four DER activating ligands. However, homology modeling shows that the conserved K48 in DER ligands will make a salt bridge with E379 and will not cause a charge repulsion with K415 in DER.

In the homology model of Spitz in complex with DER we made use of the existing crystal structure of TGFA in complex with EGFR. While the backbone structure was left unchanged the side chains were replaced by those of Spitz and DER, whereby optimal rotamers of the side chains were calculated by SCWRL (22) and checked by WHATIF (23). Overlay of the backbone structure obtained from the recently published crystal structure of Spitz (24) with that of EGF in complex with ErbB1 showed that both ligands adopt a mostly similar folding, except for the backbone structure of the B-loop (Figure 7). This mainly results from the fact that the Spitz B-loop is two residues longer than that of EGF. As a consequence modeling Spitz on the TGFA backbone will introduce errors particular in the B-loop structure.

The difference in backbone structure of the B-loop of Spitz and EGF alone, however, does not explain why Spitz does not interact with ErbB1 since the complementary chimera S3E5S, in which the B-loop sequences of Spitz were exchanged for the corresponding sequences of EGF, did not interact with the human ErbB1.

We succeeded in designing a Spitz-EGF chimera, E3S5E, with binding affinity for human ErbB1 and DER, which was able to induce MAPK activation in both DER overexpressing S2DER cells and human ErbB1 overexpressing HER14 cells. The relative binding affinities of E3S5E for DER and ErbB1 are however not very high. Remarkably, based on the primary amino acid sequence and comparison of the crystal structures and homology models of ligand-receptor complexes, we are still unable to understand why Spitz does not bind to ErbB1. Now we found that E3S5E is a low affinity ligand for both DER and ErbB1 attempts can be made to model the interaction between E3S5E and ErbB1, and E3S5E and DER, based on the available experimental crystal structure of TGFA in complex with ErbB1.

ORIGINAL INTEREST

It was the initial intention of the above study to obtain a Spitz/EGF chimera with high binding affinity for both ErbB1 and DER. Based on the initial assumption that Argos could act as a receptor antagonist by inhibiting binding of Spitz, we planned to design a humanized version of Argos that could act as a receptor antagonist for ErbB1. In order to do so, we intended to exchange domains between the above DER and ErbB1 bispecific agonist and the DER selective antagonist Argos. These considerations were based on the observation that Argos has an EGF-like domain, and the studies by Jin et al. (25) had shown that Argos binds directly to DER and prevents Spitz-induced DER dimerisation and activation. Furthermore, Howes et al. (26) had demonstrated that the Argos B-loop and linear C-terminal region are essential for the antagonistic activity of Argos, and that the C-loop of Argos could be exchanged for that of Spitz. Recent studies have shown that Argos most likely acts by scavenging Spitz, rather than by binding to DER (24). Still studies on the binding affinity of Spitz-EGF chimeras are interesting, since they may give information on the evolutionary diversification of ligands in their ability to bind their receptor.

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9

General discussion

9.1 Introduction

The ErbB signaling network is involved in the development of many tissues in the human body. The four receptors of the human ErbB family are each activated by a different subset of in total eleven ErbB ligands. Upon ligand binding receptors dimerize resulting in transphosphorylation and subsequently the activation of several signal transduction pathways. The kinase-deficient ErbB3 is dependent on the orphan ErbB2 co-receptor to form an active dimeric complex (1). This thesis focuses on the molecular basis that underlies the affinity of the ligand-receptor interaction, as well as on the ErbB specificity and mitogenic potential of their ligands. Based on our results and those of others, we conclude that affinity is mediated by a large number of residues that are directly involved in receptor binding, collectively referred to as positive constraints (2). ErbB selectivity, however, is mainly determined by residues that are not essential for high affinity binding to their cognate receptor but that even in presence of sufficient positive constraints prevent or strongly impair the unintended binding to other ErbB receptors. These residues, which we collectively refer to as negative constraints, can prevent receptor-ligand interaction either by charge repulsion, steric hindrance or by changing the structure of the ligand. The relation between receptor affinity and mitogenic potential of ligands is very complex and is not completely understood. On many cells, there appears no direct relation between ligand affinity and mitogenic activity. For ErbB1-binding ligands the mitogenic activity has been shown to depend in addition on the on/off rate of the ligand-receptor complex (3), the pH sensitity of the internalised receptor-ligand complex and on the rate of ligand depletion from the medium as a result of receptor binding and internalisation (4). Heterodimerization of ErbB1 with ErbB2 decreases the rate of internalisation of ErbB1-bound ligands (5) and promotes ErbB1 recycling, but it remains unclear if this fully explains the enhanced mitogenic effect of ErbB1/ErbB2-bound ligands. The difference between receptor affinity and mitogenic activity of ErbB3 and ErbB4 binding ligands is even much less clear, and will be discussed here.

It is remarkable that during evolution low affinity ErbB ligand have maintained themselves. It has been proposed that low affinity ligands might preferentially induce the formation of ErbB2-containing heterodimers, suggesting that there must be evolutionary pressure on the maintenance of such heterodimeric signaling. This can particularly be understood for ErbB3 binding ligands, assuming that low affinity ligands preferentially induce biologically active ErbB2-ErbB3 heterodimers, while high affinity ligands may preferentially induce biologically inactive ErbB3 homodimers (6). We will describe a model in which signaling through ErbB2-ErbB3 heterodimers is determined by the ratio and distribution of ErbB2 and ErbB3 on the cell surface, as well as the affinity of the ligand for ErbB3. Finally, activation of ErbB4 signaling depends in a complex manner on the ligand binding affinity and receptor heterodimerisation with ErbB2. In addition, however, ErbB4

signaling is dependent on its specific isoform and signaling mechanism (canonical vs non-canonical), as well as on the presence of TACE and γ-secretase enzymes that can cleave the ErbB4 receptor. This chapter discusses the above-mentioned conclusions and provides suggestions for further research.

9.2 Molecular basis of binding affinity and specificity

The interaction of EGF and TGFA with ErbB1 has been studied extensively by a variety of techniques. Crystallographic data of EGF and TGFα in complex with ErbB1 (7, 8) have revealed the amino acids that are directly involved in receptor binding, while mutagenesis studies have indicated the relative contribution of individual residues in terms of receptor binding affinity (9). This type of studies has resulted in the identification of positive constraints in ligand-receptor interaction. On the other hand, domain-exchange strategies have provided essential information for our understanding of the ligand specificity of ErbB receptors, and have resulted in the identification of so-called negative constraints in ligand-receptor interaction. This concept has been based initially on our observation that human TGFA is a high affinity ligand for both the human and the chicken EGF receptor, while human EGF is only a high affinity ligand for human ErbB1, but has a 10-50 fold lower affinity for the chicken EGF receptor (10). Our domain-exchange studies have shown that exchange of R45 from EGF into A45 from TGFA is sufficient to make EGF a high affinity ligand for the chicken EGF receptor. Since the R45A mutation does not affect the affinity of EGF for ErbB1 (10), it must be concluded that R45 does not significantly contribute to the binding affinity of EGF to its receptor, but can impair the binding to other, related receptors.

The resulting concept of negative constraints gained further relevance by our observation that T1E, a chimera between EGF and TGFA, does not only bind ErbB1, but has broadened its receptor specificity by binding to ErbB3 and ErbB4 (11). Subsequent phage display approaches resulted in the identification of mutant forms in which positive constraints were enhanced and negative constraints depleted. In the present studies we have focused on the role of the C-terminal linear region, and thereby identified a mutant form of EGF, designated panerbin (T1E/YYDLL), which binds with high affinity to all three ligand binding ErbB receptors (2). Subsequent homology modeling of various phage display selectants provided an explanation why some residues act as a positive and other as negative constraint in binding to the various ErbB receptors.

Based on a combination of these various approaches, we have been able to divide the residues in EGF-like growth factors into four categories. First of all, the conserved residues that are essential for the tertiary structure: the six cysteines, as well as G18 and G39. Secondly, the less conserved amino acids that are not directly involved in receptor binding, but play a role in the

secondary structure formation. These include Y22 and Y29 that are essential for proper beta-sheet formation of the B-loop (8). Thirdly, the residues that are directly involved in ErbB1 binding (positive constraints), some of which are the highly conserved (Y13, L15, R41, L47) , while others (E40, Y44, R45 and K48) are less strict (9). The fourth group of residues are those that are not essential for high affinity binding but are conserved because they are involved in ErbB specificity by preventing the unintended binding to other ErbB molecules (negative constraints): S2D3 in EGF, E26 in TGFA and R45 in EGF. We could not identify residues that can be mutated at will and are only essential for the proper amino acid spacing, and therefore the conformation, of the looped regions. P7, V19, E24, D27 and V34 are examples of residues that are not in direct contact with the receptor and appear not essential for high affinity binding (9). Still, these residues are well conserved among vertebrate EGF orthologues. Therefore we must conclude that in a small molecule like EGF every amino acid plays a functional role.

The recent availability of the crystal structure of liganded and unliganded ErbB receptors has made it possible to understand the molecular action of negative constraints by homology modeling. Our experimental data show that R45 of EGF is a negative constraint for binding to ErbB3 and ErbB4, and homology modeling shows that this may be due to charge repulsion with K415 and K418, respectively. Since R45 is not essential for high affinity binding of EGF to ErbB1, it appears that the conserved presence of a positive residues at this position in EGF is important for obtaining the proper receptor specificity. AREG is an ErbB1 specific ligand, while its evolutionary homologue HB-EGF binds both ErbB1 and ErbB4. Interestingly, AREG has an Arg on a similar position as R45 in EGF, while HB-EGF has not. This indicates that during evolution the introduction or release of negative constraints may have resulted in ligands with the requested receptor specificity.

Our observation that R45 of EGF causes a charge repulsion with K415 and K418 in ErbB3 and ErbB4, respectively, made us curious for the mechanism by with R45 of EGF prevents binding to the chicken EGF receptor (CER). Therefore we looked for positively charged amino acids in CER that would come in close contact with R45 in EGF. A putative candidate is K415 in CER which might cause charge repulsion with R45 in human EGF. Figure 1 shows EGF in complex with ErbB1 and panerbin in complex with ErbB3. K415 in CER, the corresponding residue for G441 in ErbB1 and A439 in ErbB3, is orientated close to the position of K415 in ErbB3. Thus R45 in human EGF probably prevents CER binding by causing charge repulsion with K415 in CER. This hypothesis can be substantiated by measuring the distance between K415 of CER and A45 of the EGF/R45A mutant, in a homology model of CER in complex with the EGF/R45A. Eventually, the hypotheses can be tested experimentally by mutating K415 in CER.

The observation that EGF in which the N-terminal linear region has been replaced by TGFA sequences (T1E) is able to bind ErbB3 and ErbB4, indicates that the linear N-terminus of EGF contains a negative constraint

for binding to ErbB3 and ErbB4. Our studies have indicated that the S2/D3 sequence in EGF impairs binding to ErbB3, most likely because these two residues do not provide the proper ligand conformation for receptor binding. It has been hypothesized that triple beta-sheet formation between the N-terminal linear region and the B-loop of the ligand is essential for binding to ErbB3, but not to ErbB1 (12, 13). These requirements are much better met by the H2/F3 sequence in TGFA than with S2/D3 in EGF, in agreement with protein conformation predictions by the Chou-Fasman algorithm. Again, since S2/D3 in EGF is not essential for ErbB1 binding, the role of the N-terminal linear region in EGF appears primarily to prevent the unintended binding to other receptors. Further research will have to indicate to what extent the N-terminal linear region plays a role in binding selectivity between ErbB3 and ErbB4.

Mutation studies have indicated that E26 in the B-loop of TGFA forms the primary negative constraint for binding to ErbB3. Homology modeling shows that E26 would induce steric hindrance with M97 of this receptor, whereas it can act as a positive constraint for binding to ErbB1 by formation of

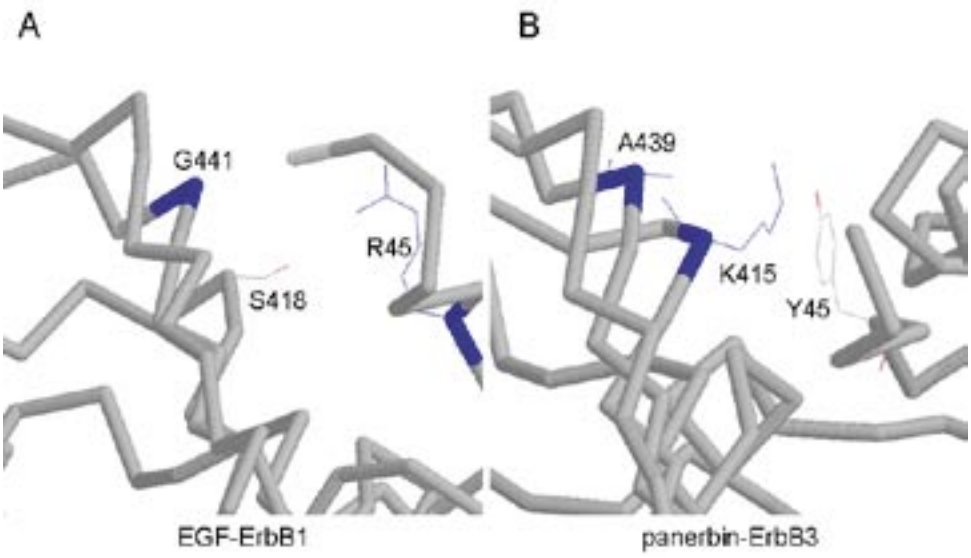


Figure 1. Involvement of K415 in CER in a charge repulsion with R45 in hEGF. A. Homology model of EGF in complex with ErbB1. R45 in EGF is indicated as well as the ErbB1 residues S418 and G441, the corresponding residues of K415 in ErbB3 and K415 in CER. B. Homology model of panerbin (T1E/YYDLL) in complex with ErbB3. K415 in ErbB3 is indicated as well as A439, the corresponding residue of K415 in CER, and Y45, the corresponding residue of R45 in human EGF. K415 in CER, the corresponding residue for G441 in ErbB1 and A439 in ErbB3, is orientated close to the position of K415 in ErbB3.

a salt bridge with R124 of this receptor. In comparison, ErbB4 contains a bulky Phe at position 97, but this residue is able to form another rotamer than M97 of ErbB3 and will thereby not cause steric hindrance. Preliminary data show that E26 does not act as a receptor-selective negative constraint, since it also reduces ligand affinity for ErbB4. Using a series of protein A-tagged EGF/TGFA chimeric ligands, it is shown in Figure 2 that T1E displays the highest binding affinity for ErbB4 (IC50 = 40 ng/ml), followed by T3E (IC50 = 300 ng/ml) and T4E, qualitatively very similar to our previous observations on ErbB3 (11). T3E and T4E differ only in their B-loop, which contains EGF sequences in T3E and TGFA sequences in T4E. In order to study to what extent the difference in affinity between T3E and T4E results from E26, we mutated this residue in T4E for the corresponding Leu from EGF. Figure 2 shows that T4E/E26L has much higher binding affinity for ErbB4 than T4E itself, indicating that E26 not only acts as a negative constraint for ErbB3, but also for ErbB4. In contrast to ErbB3, however, E26 appears unable to fully prevent ErbB4 binding. This is indicated by our observation that TGFA/YYDLL, which contains E26 as the only known negative constraint, has detectable affinity for ErbB4, but not for ErbB3. Interestingly, E26 is evolutionary conserved in the ErbB4 binding ligand BTC (Figure 9), which indicates that the effect of this negative constraint can be overcome by a sufficiently large number of positive constraints for this receptor. Thus our data show that ErbB3 and ErbB4 largely recognize similar negative constraints (E26; R45), but ErbB3 appears much more sensitive for the inhibitory effects of these residues than ErbB4.

While positive constraints form the major basis for binding affinity, negative constraints appear to control binding selectivity. Figure 3A illustrates schematically how mutation of a positive constraint can lead to a decrease in receptor binding affinity, because important interactions with the receptor

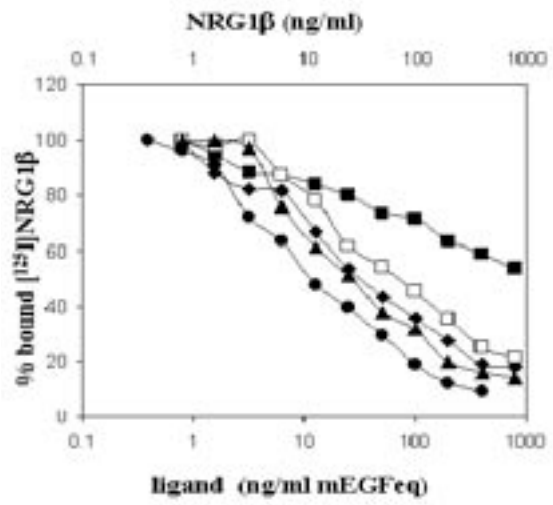


Figure 2. Ligand binding displacement assay of [^{125}I-]-NRG1β on T47-14 cells. Cells were treated with increasing concentrations of NRG1β (—●—), protein A-T1E (—▲—),protein A-T3E (—◆—), protein A-T4E (—■—) and protein A-T3E/E26L (···□···). The concentration of the protein A-tagged TGFA-EGF chimeras are indicated in mEGF binding equivalents for binding to ErbB1 overexpressing HER14 cells. Values of bound radiolabeled ligand without competition of unlabeled ligand was set on 100%. Results are the mean of at least two independent experiments performed in duplicate.

are lost. For instance mutation of L47 in EGF to Ala results to a dramatic decrease of ErbB1 affinity (14), because of depletion of an important positive constraint. Depletion of a single negative constraint in a ligand, however, can enhance the affinity for its receptor many fold without affecting the number of putative interactions between ligand and receptor. The E26L mutation in T4E and R45Y mutation in T1E strongly enhanced affinity for ErbB3 and ErbB4. One could argue that both mutations exchange a negative constraint for a positive constraint and that therefore the enhanced affinity does not only result from a release of a negative constraint. For future research it will be interestingly to mutate these negative constraints to Ala, since this will in general not result in introduction of a positive constraint. Furthermore it will be interestingly to study the effect of artificially introduced negative constraints in ErbB specificity, e.g. by local introduction of bulky Trp residues.

Van de Poll et al. (15) have studied the minimal requirements for high affinity ligand binding to ErbB1. In their study they showed that an EGF-like repeat from the Drosophila Notch protein, linked to the N- and C-terminal linear sequences of human EGF, did not show measurable binding affinity for

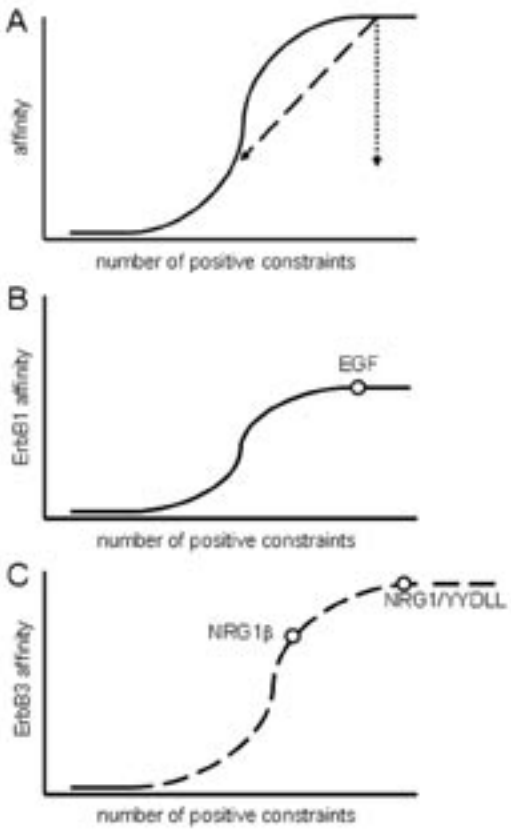


Figure 3. Relation between ErbB-ligand binding affinity and the number of interaction sites. A. Dashed arrow indicates the effect of introduction of a negative constraint. The solid arrow indicates the effect of mutation of a positive constraint. Deletion of a positive constraint results in a decrease in binding affinity due to a decrease in the number of interactions between ligand and receptor, whereas a negative constraint decreases affinity without affecting the number of interaction sites. B. EGF has the maximum affinity for ErbB1. No mutations in EGF have resulted in significant increase of ErbB1 affinity. C. NRG1β does not have maximal affinity for ErbB3, since introduction of additional positive constraints by replacement of the NRG1β linear C-terminal region by the YYDLL sequence of panerbin results in a strong increase of ErbB3 affinity.

ErbB1. However, introduction of only six amino acids from human EGF, which are known to be important for ErbB1 binding, was sufficient to make Notch a high affinity ligand for ErbB1. The introduced residues from EGF included Y13 in the A2-loop, M21, Y22 and A29 in the B-loop, N31 in the hinge and E40 in the C-loop. Out of these six residues only Y13 is present in Spitz. However, in the chimera S3E5S, which does not bind ErbB1, all these residues are present with the exception of E40. Unlike the Notch mutants studied, however, S3E5S lacks residues in the C-terminal linear region, including L47, that are essential for high affinity binding. It therefore appears that Spitz cannot bind ErbB1, because important positive constraints are lacking.

NRG1β lacks all the six residues mentioned above, but has a Phe as a functional substitution for Y13, and an Asp for E40. However, the NRG1β-panerbin chimera N3P4N6P has in addition the three residues from the B-loop of EGF, while its Lys in the hinge region is also present in the ErbB1-specific ligand AREG. Since N3P4N6P has an optimal C-terminal linear region for binding to ErbB1, it therefore appears that its complete lack of affinity for ErbB1 does not result from the absence of positive constraints. We must therefore conclude that NRG1β most likely lacks binding affinity for ErbB1, because of the presence of so-far unidentified negative constraints. From an evolutionary point of view it makes sense that Drosophila Spitz and EGF do not bind to each other's receptor, since they are present in highly diverted species.

9.3 The molecular basis of mitogenic activity

Binding affinity and mitogenic activity are not necessarily correlated, as has been studied in particular detail for ErbB1 binding ligands. Low affinity EGF mutants may have similar mitogenic activity as wild-type EGF, particularly on ErbB1 overexpressing cells (16-18). Other mutants have similar binding affinity as EGF but show enhanced mitogenic activity, thus acting as so-called superagonists. We have shown that the EGF/TGFA chimeras E3T and E4T induce mitogenic activity at a ten-fold lower concentration than wild-type EGF or TGFA, in spite of the fact that they have similar receptor binding affinity for ErbB1 and induce the same extent of receptor tyrosine kinase activity (19). BIAcore experiments have shown that these superagonists have enhanced on/off rates (3) suggesting that the frequency whereby the ligand binds its receptor determines the mitogenic activity of a ligand, more than the mean time it occupies the receptor. Upon activation of ErbB1 by EGF the EGF-ErbB1 complex is internalized via clathrin-coated vesicles and continues to signal in the endosomes until both ligand and receptor are degraded in the lysosomes. TGFA, on the other hand, also induces ErbB1 internalization but rapidly dissociates from the receptor due to the low pH in the endosomes, resulting in receptor recycling to the cell surface where it can be reactivated. So EGF signals from the endosomes for a relatively long period of time after which

the receptor is degraded, whereas TGFA signals for a relatively short period of time but the receptor molecules can signal more frequently because of receptor recycling. Still, both the binding affinity and mitogenic activity of EGF and TGFA are similar. The superagonist E4T shows enhanced ErbB1 recycling which correlates with decreased receptor ubiquitination and degradation (20). E4T has a pH sensitivity of binding which is intermediate between EGF and TGFA, and therefore it has been proposed that the superagonistic behavior can be explained by the relatively long signaling duration along the endocytic routing, similar to EGF, in combination with recycling of the receptor to the cell surface, similar to TGFA (5).

It has been shown that growth factor-induced cell cycle progression of quiescent cells occurs in two distinct phases of signaling. The first phase stimulates the cells to leave the G_0 phase and requires activation of MAPK and c-Myc. The second phase occurs 8 hours later and drives cells through the restriction point of late G_1-S phase, a process which requires activation of the PI3K-Akt pathway (21, 22). As a result of receptor-mediated internalization high-affinity ligands such as EGF are depleted much more rapidly from the extracellular medium than low affinity ligands (4). As a consequence, the mitogenic activity of EGF on ErbB1 overexpressing cells is limited by substantial degradation of internalized ligand in the period before cells pass the restriction point in the late G1-S phase. This has been evidenced by the observation that the concentration required for half-maximum growth stimulation by EGF decreases upon increasing the volume in which the assay is carried out. The observation that superagonists have higher on/off rates than wild-type ligands, could result in reduced ligand internalisation and degradation rates, but experimental evidence for this hypothesis is still lacking so far (3).

Since ErbB3 and ErbB4 are not internalized upon ligand binding, pH sensitivity of binding and ligand depletion from the medium do not appear to control the mitogenic activity of their ligands. Since ErbB3 is dependent on ErbB2 for forming a mitogenic complex, the efficacy by which a ligand can form ErbB2-ErbB3 heterodimers will largely determine the mitogenic potential of a ligand. For the relationship between ErbB3 affinity and mitogenic activity on ErbB2 and ErbB3 overexpressing cells we propose two alternative models. The first model is a dynamic model (Figure 4), according to which the ligand binds an ErbB3 receptor after which the complex recruits either an ErbB2 or another ligand-bound ErbB3 receptor. Crucial for this model is that the liganded ErbB3 receptor is able to form biologically inactive homodimers or is stable as a monomer. We have observed that NRG/YYDLL has a higher binding affinity for ErbB3 than wild-type NRG1β, while the latter has higher mitogenic activity on cells containing both ErbB2 and ErbB3. This observation could be explained in the dynamic model if NRG/YYDLL with its high affinity for ErbB3 forms more ErbB3 homodimers, while NRG1β with its lower affinity for ErB3 gives rise to more ErbB2-ErbB3 heterodimers. However, besides the fact that ErbB3 homodimers have not been identified experimentally, this model can

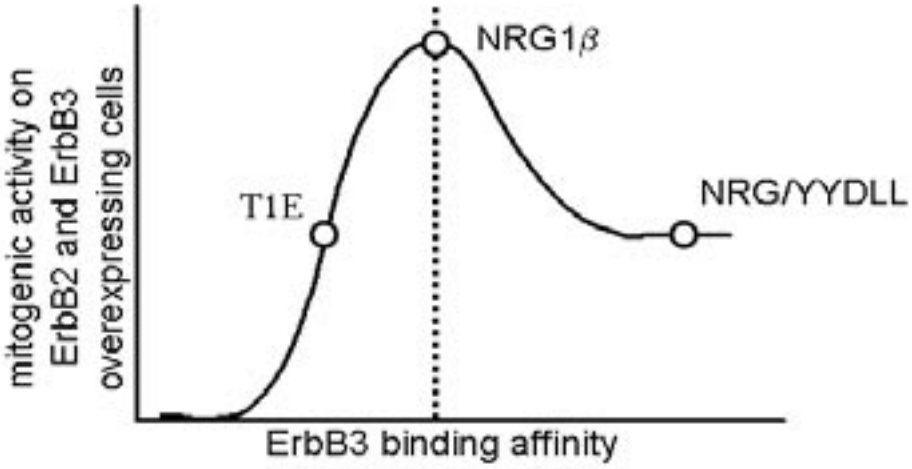


Figure 4. Relation between ErbB3 binding affinity and the mitogenic potential on cells expressing ErbB3 and ErbB2 according to the dynamic model. Low affinity ErbB3 ligands depend on the presence of ErbB2 to form a stable dimer. High affinity ErbB3 ligands do not depend on ErbB2 to form a stable complex with ErbB3, whereas low and intermediate ErbB3 binders preferentially form signaling ErbB2-ErbB3. It is assumed that high affinity ErbB3 binders are also able to form inactive ErbB3 monomers or homodimers. The mitogenic activity of a ligand on ErbB2 and ErbB3 overexpressing cells depends on the ability to form ErbB2-ErbB3 heterodimers. T1E is a low affinity ligand for ErbB3 and therefore depends on ErbB2 to form a stable ErbB2-ErbB3 complex, which is mitogenically active. Still the affinity of T1E for ErbB2-ErbB3 heterodimer is not high enough the form the maximal number of ErbB2-ErbB3 heterodimers. NRG/YYDLL has a high affinity for ErbB3 and does not depend on ErbB2 to form a stable ligand ErbB3 complex. In fact the affinity of NRG/YYDLL for the ErbB2-ErbB3 heterodimer is for unknown reasons lower than for ErbB3 alone. Therefore NRG/YYDLL will form ligand-ErbB3 complexes in addition to ErbB2-ErbB3 heterodimers. ErbB3 homodimers or monomeric ligand ErbB3 complexes do not give a mitogenic response, since ErbB3 has an inactive kinase. Wild-type NRG1β has the optimal mitogenic capacity, since it has the optimal binding affinity for the ErbB2-ErbB3 heterodimer. It is able to form a lot a ErbB2-ErbB3 heterodimers while formation of ErbB3 homodimers or ligand-ErbB3 monomeric complexes is not at the expense of the number of ErbB2-ErbB3 heterodimers being formed.

not readily explain why NRG/YYDLL binds on D3 cells with higher affinity than on D23 cells. Alternatively, the data can be explained in terms of a preformed dimer model. Junttila et al. (23) have shown that ligand-independent preformed ErbB2-ErbB3 heterodimers exist on the surface of SKBR3 cells. If the majority of receptors on D23 cells would be present as such preformed ErbB2-ErbB3 heterodimers, with only few free ErbB3 monomers remaining, this could explain why both NRG/YYDLL and NRG1β have similar binding affinity on these cells. However, this model appears unable to explain why, in

spite of this similar binding affinity, NRG1β has higher mitogenic activity than NRG/YYDLL on these cells. In conclusion, these two models can only partly explain the observed discrepancy between binding affinity and mitogenic activity of NRG1β and NRG/YYDLL for cells containing only ErbB3 and cells containing both ErbB2 and ErbB3 receptors.

In contrast to ErbB3, ErbB4 can form signaling homodimers and the mitogenic potential, measured by [^{3}H]-thymidine incorporation on ErbB2-negative ErbB4 overexpressing T47-14 cells, largely correlates with the binding affinity. In the presence of ErbB2 cell survival, measured in an MTT assay on ErbB2 and ErbB4 expressing D24 cells, correlates with binding affinity to ErbB4, probably because ErbB4 does not depend on ErbB2 in order to form a signaling dimer. T1E/YYDLL binds with higher affinity to ErbB4 homodimers than to ErbB2-ErbB4 heterodimers, but it is unclear whether at the low concentrations used in mitogenic assays this ligand preferentially signals through ErbB4 homodimers or through ErbB2-ErbB4 heterodimers. From an evolutionary point of view our models, for the relationship between ErbB affinity and mitogenic activity, might explain the occurrence of the alpha and beta isoforms of NRG1 and NRG2, which differ in their affinity for ErbB3 and ErbB4 but not in receptor specificity. The alpha isoforms have low affinities for ErbB3 and ErbB4 alone and show higher binding affinities in presence of ErbB2 (25), suggesting that the alpha isoforms preferentially signal through ErbB2-ErbB3 or ErbB2-ErbB4 heterodimers rather than through ErbB4 homodimers. On cells with a surplus of ErbB2 over ErbB3 receptors, high affinity ligand will give an optimal mitogenic response, but on cells with a surplus of ErbB3 over ErbB2 receptors low affinity ligands are preferred, since these will not give rise to biologically inactive ErbB3 monomers of homodimers.

Mitogenic stimulation through ErbB4 is further complicated by the presence of different isoforms of this receptor, which can either signal through tyrosine phosphorylation or by cleavage of a soluble intracellular domain, which can act as a transcriptional cofactor (26). Overexpression studies in 32D cells have demonstrated that the JM-a CYT-2 and the JM-b CYT-1 isoforms of ErbB4 have the highest potential in NRG1β-induced cell survival assays (27). The JM-a CYT-2 isoform, however, also promotes ligand-independent signaling by a mechanism involving both ligand-independent tyrosine phosphorylation and proteolytic generation of the intracellular domain (27). The cleavable JM-a isoform is more abundantly expressed in breast cancers than the uncleavable JM-b isoform. Expression of the ErbB4 intracellular domain correlates with poor prognosis for breast cancer patients in comparison with cell surface ErbB4 expression (28). Finally it has been shown that BTC and NRG1β induce different phosphorylation patterns of ErbB4 and might therefore induce a different level of mitogenic activity (29). In conclusion, the mitogenic activity mediated by ErbB4 depends on ligand affinity, the presence of ErbB2, the induced receptor phosphorylation pattern and the ErbB4 isoform.

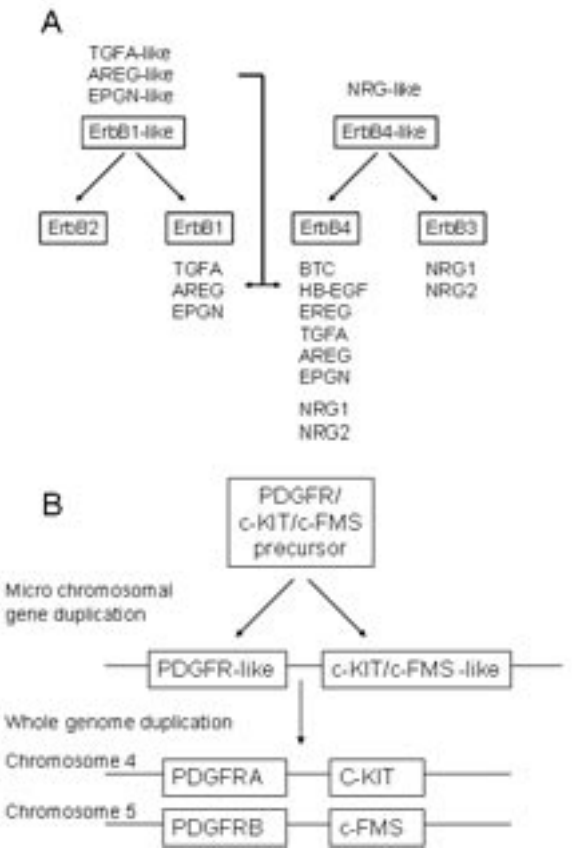
It is intriguing that during evolution low affinity ligands have been maintained for for all ligand binding ErbB receptors. For ErbB1 EREG acts

as a low affinity ligand, while NRG1α and NRG2α are low affinity ligands for ErbB3 and ErbB4. As mentioned it has been speculated that for ErbB2-ErbB3 heterodimers low affinity ligands are preferred, in order to avoid the formation of biologically inactive ErbB3 homodimers. In the case of ErbB4, it can be imagined that low affinity ligands will preferentially activate ErbB2-ErbB4 heterodimers, with its higher diversity of intracellular second messengers. In the case of ErbB1, low affinity ligands may be preferred on cell types with a high receptor density, in order to avoid ligand depletion in a small compartment. It remains to be investigated if these ligands show reduced affinity due to loss of positive constaints or due to introduction of negative constaints.

9.4 Coevolution of ErbB receptors and their ligands

During evolution of vertebrae two whole genome duplications have occurred. Evolution of ErbB receptors started with a single ErbB-precursor receptor and one or a few activating ligands, similarly as still present in *Drosophila melanogaster*. Our hypothesis of how ErbB receptors and their ligands may have coevolved is illustrated in Figure 5A. It is proposed that during the first whole genome duplication a single parental ErbB precursor receptor has given rise to a proto-ErbB1 receptor and a proto-ErbB4 receptor. The proto-ErbB1 receptor would probably be bound and activated by proto-TGFA, proto-AREG and proto-EPGN type of ligands, similar to the current situation in *Ciona intestinalis* (Van Leeuwen, unpublished). The proto-ErbB4 receptor would most likely be bound and activated by a proto-NRG type of ligand, also similar to the current situation in *Ciona intestinalis* (Van Leeuwen, unpublished). Following the second whole genome duplication, the duplicated proto-ErbB1 receptor evolved into a ligand-activated ErbB1 and an orphan ErbB2 receptor, with a conformation ready to dimerize with other liganded ErbB receptors. Simultaneously, the proto-ErbB4 receptor gave rise to ErbB4 and the kinase dead ErbB3 receptors.

Along with the ErbB receptors the proto-TGFA, proto-AREG and proto-NRG ligands duplicated into TGFA and BTC, AREG and HB-EGF, and NRG1 and NRG2, respectively. The duplication of proto-EPGN into EPGN and EREG happened most likely by micro-chromosomal gene duplication rather than during the whole genome duplication (30). Remarkably, duplication of the ligands for the ErbB1-like receptors resulted in ErbB1 specific ligands, e.g. TGFA, AREG and EPGN, and ligands with ErbB1-ErbB4 dual specificity, e.g. BTC, HB-EGF and EREG, in a pair-wise manner. Multiplication of proto-NRG resulted in NRG1 and NRG2, which show similar ErbB receptor specificity. Similarly, the alpha and beta isoforms of NRG1 and NRG2 bind and activate both ErbB3 and ErbB4, although with different affinities. Since the ErbB specificity of the evolutionary related NRG3 and NRG4 has not been well characterized, it is not possible to speculate on the mechanism that has put



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Gene	Chromosomal location
Receptors	
ErbB1	7p12
ErbB2	17q21.1
ErbB3	12q13
ErbB4	2q33.3-q34
Ligands	
EGF	4q25
TGFα	2p13
BTC	4q13-q22
HBEGF	5q23
AREG	4q13-q21
EPGN	4q13.3
EREG	4q13.3
NRG1	8p12
NRG2	5q23-q33
NRG3	10q22-q23
NRG4	15q24.2
PDGFR related	
PDGFRA	4q11-q13
PDGFRB	5q31-q32
c-KIT	4q11-q13
c-FMS	5q31-q32

Figure 5. A. Hypothetical model for the coevolution of ErbB receptors and their ligands. Evolutionary pressure on the receptor specificity of ErbB ligands has resulted in the introduction of negative constraints in the ligands. It is assumed that after the first whole genome duplication two ErbB receptors have evolved, an ErbB1-like and an ErbB4 like precursor. The ErbB1-like receptor was probably activated by a TGFA-like, an AREG-like and a EPGN-like ligand, while the ErbB4-like receptor was activated by a NRG-like precursor. Following a second whole genome duplication, the ErbB1-like precursor evolved into the ErbB1 and the orphan ErbB2 receptor, while the ErbB4-like receptor gave rise to ErbB4 and the kinase death ErbB3. The TGFA-like, AREG-like and EPGN-like ligands each developed into an ErbB1-specific and an ErbB1/ErbB4 bispecific ligand, whereby bispecificity resulted from a release of negative constraints for ErbB4. EGF is not included since this protein belongs to the LDL receptor family and has most likely followed a different route during evolution. NRG3 and NRG4 are also not included, since there is little known about their affinities and ErbB specificities. B. Evolution of PDGF receptors and c-KIT/c-FMS. C. Chromosomal localizations of human ErbB receptors, their ligands and PDGF receptor-related genes. Note that EPGN, EREG, AREG and BTC are located next to each other on chromosome 14q13. Similarly PDGFRA and c-KIT, as well as PDGFRB and c-FMS are neighboring genes on chromosome 4q11-q13 and 5q31-q32, respectively.

evolutionary pressure on the maintenance of these two ligands.

Since during evolution the proto-ErbB1 specific ligands all three diverted into an ErbB1 specific ligand and a ligand that is bispecific for ErbB1 and ErbB4, it is tempting to speculate that one of the duplicate ligands lost its negative constraints for binding to ErbB4, while maintaining those for ErbB3. It would be very interesting to identify the ErbB4-specific negative constraints that are still present in TGFA, AREG and EPGN, but lost in BTC, HB-EGF and EREG. Domain-exchange studies of the intercysteine domains of the EGF-like domains between the evolutionary related pairs TGFA and BTC, AREG and HB-EGF, and EPGN and EREG will show if the ErbB1-specific ligands share the same negative constraints for binding to ErbB4.

In order to substantiate the hypothesis of the duplication of ErbB genes and their ligands, we made a comparison with the evolution of platelet-derived growth factor receptor (PDGFR)-related genes (Figure 5B). Evolution of the mammalian PDGF alpha-receptor and PDGF beta-receptor genes and their evolutionary related genes c-KIT and c-FMS probably started with a micro-chromosomal gene duplication generating a PDGFR-like precursor and a c-KIT/c-FMS-like receptor (31). Following the whole genome duplication the PDGFR-like and the c-KIT/c-FMS-like receptor gave rise to the PDGFRA and PDGFRB on the one hand, and c-KIT and c-FMS on the other hand which are still present in the mammalian genome (32). In addition to primary amino acid sequence homology, the duplication of neighboring genes is another clue for the suggestion that two genes have been derived from duplication of a common ancestor. Unlike the PDGFR-related genes, however, the evolutionary related ErbB receptors (ErbB1 and ErbB2; ErbB3 and ErbB4) and EGF-like growth factors (TGFA and BTC; AREG and HB-EGF; EPGN and EREG) do not show chromosomal co-localization. Figure 5C shows the chromosomal locations of human ErbB receptors and their ligands in relation to PDGFR-related genes.

9.5 Why does NRG1β not bind ErbB1?

The negative constraints we have identified so far, are residues in ErbB1 ligands that prevent the interactions with the NRG receptors ErbB3 and ErbB4. These negative constraints explain why EGF and TGFA do not bind ErbB3 and ErbB4, but it remains unclear why NRG1β does not interact with ErbB1. Based on the crystal structure TGFA in complex with ErbB1, we made homology models of NRG1β in complex with ErbB3 and ErbB4. These models showed that NRG1β can form similar positive constraints with these receptors as TGFA with ErbB1, while in addition no evidence was found for steric clashes or charge repulsion that would prevent receptor binding. Thus based on these homology models no argument was found why NRG1β does not interact with ErbB1.

Domain exchange studies by Barbacci et al. (33) showed that introduction of each intercysteine sequence of NRG1β into EGF resulted in a dramatic loss of ErbB1 affinity. This suggests that all intercysteine domains of NRG1β either lack an important positive constraint or have a negative constraint which prevents ErbB1 interaction. In Chapter 3 we discussed panerbin, an EGF-TGFA chimera that has lost all negative constraints and therefore interacts with ErbB1, ErbB3 and ErbB4. Subsequent domain exchange studies between NRG1β and panerbin (Chapter 7) demonstrated that the A1-loop (sequence between the first and the second cysteine) and C-loop (sequence between the fifth and the sixth cysteine) of NRG1β are involved in preventing interaction with ErbB1. It is currently unknown, however, whether this results from negative constraints for ErbB1 in the A1-loop of NRG1β, or that the identified residue D11 in the A1-loop of EGF is an essential positive constraint for ErbB1 interaction. Interestingly, in our homology models most of the residues present in the A1-loop and C-loop are oriented away from the receptor molecule and are therefore not directly involved in receptor-ligand binding. It can be speculated that the A1-loop and C-loop play a, so far not recognized, subtle role in the conformation of the ligand and thereby directly affect receptor-binding affinity. Furthermore it cannot be excluded that upon introduction of the A1-loop or C-loop of NRG1β, panerbin loses the proper conformation for binding to ErbB1, although high affinity for ErbB4 is maintained.

9.6 Design of ErbB3 and ErbB4 specific ligands

Based on their specificity, naturally occurring ErbB ligands can be divided into three groups; ligands that bind exclusively ErbB1, the neuregulins that interact with ErbB3 and ErbB4 and ligands that bind to ErbB1 and ErbB4 but not to ErbB3. It has been claimed that NRG3 and NRG4 are specific for ErbB4, but these ligands have not been characterized in detail. Some reports show activation of ErbB2-ErbB3 heterodimers by these two ligands as well (34). Furthermore it appears that these two ligands bind ErbB4 with only very low affinity (25). This implies that ligands which specifically bind ErbB3 or ErbB4 do not occur in nature. Still, designed ErbB3 and ErbB4 specific ligands hold great promise as therapeutic drugs (see below).

ErbB3 and ErbB4 specific ligands can theoretically be developed by introduction of ErbB4 and ErbB3-specific negative constraints in NRG1β, respectively. However, homology modeling studies have not given any indication for the presence of such receptor-specific constraints in NRG (35). Alternatively, EGF-based ligands are available that discriminate between ErbB3 and ErbB4, and these could be used as a basis for development of ErbB3 or ErbB4 specific ligands, provided that the affinity for ErbB1 can be eliminated. Emphasis in our studies have so far been on the identification of negative constraints for ErbB3 and ErbB4, and therefore putative ErbB1-specific

negative constraints have not been identified yet. Such negative constraints for ErbB1 could be identified e.g. by investigating which intercysteine domain of NRG has to be introduced into BTC to prevent ErbB1 binding. Since both NRG1β and BTC bind ErbB4, the NRG1β-BTC chimeras will most likely retain their high binding affinity for ErbB4, which will be helpful for purification and quantification of biologically active fractions of these chimeras. Subsequent introduction of ErbB1-specific negative constraints into BTC will result in the generation of an ErbB4 specific ligand.

Our previous studies have indicated that not only the ligand's C-terminal, but also the N-terminal linear region plays an important role in ErbB selectivity. By introducing the optimal sequence requirements for ErbB3 binding in both the N- and C-terminal linear regions we designed an EGF mutant, designated WVR/EGF/IADIQ, which shows high affinity for ErbB3 and ErbB1, but only very low affinity for ErbB4 (Figure 6). This ligand could be used as a starting point for designing ErbB3 selective ligands, by introducing negative constraints for ErbB1 and further reducing the affinity for ErbB4.

Design of ligands that specifically interact with either ErbB3 or ErbB4 will attribute greatly to our understanding or ErbB specificity but can also

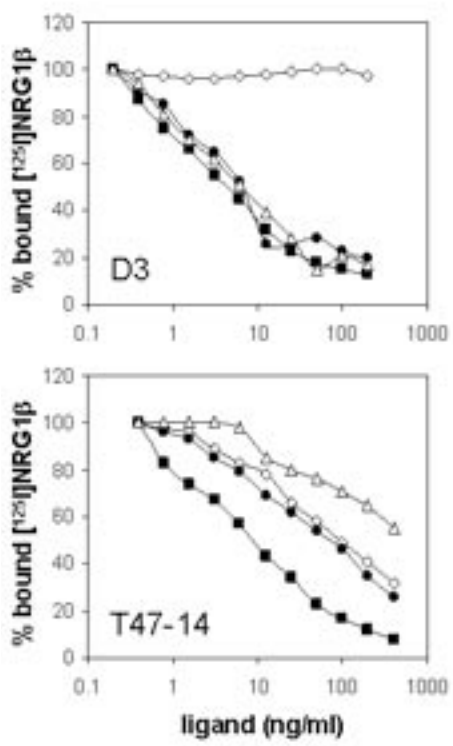

Figure 6. Binding affinity of mutant EGF-like growth factors for ErbB3 and ErbB4. (**A**) Ligand binding displacement analysis on ErbB3 overexpressing D3 cells treated with 1 ng/ml [^{125}I]-NRG1β. (**B**) Ligand binding displacement analysis on ErbB4 overexpressing T47-14 cells treated with 1 ng/ml [^{125}I]-NRG1β. Cells were incubated with increasing amounts of the unlabeled NRG1β (—■—), EGF/YYDLL (—◇—), T1E/IADIQ (—●—) and VWR/IADIQ (—△—). Values of bound radiolabeled ligand without competition of unlabeled ligand were set on 100%. Results are the mean of at least two independent experiments performed in duplicate.

be of great therapeutic value. *In vivo* treatment with recombinant NRG1 has been shown to improve cardiac function and survival in animals with ischemic, dilated and viral cardiomyopathy (35), while reduced ischemic-induced brain damage has been observed in rats (36). ErbB3 and ErbB4 have different functions in cardiac and brain development, as known from receptor-specific knock-out mice. Therefore, *in vivo* treatment with NRG may result in unintended side effects because it activates both ErbB3 and ErbB4. It would therefore be of great interest if receptor-specific agonistic ligands can be designed for treatment of patients with the above diseases.

From a scientific point of view administration of ErbB3 and ErbB4-selective ligands can be used in order to study the individual roles of ErbB3 and ErbB4 in developmental processes or in *in vitro* differentiation assays of for instance neuronal progenitor cells. Because of the role of NRG in the neuro-muscular junction and in oligodendrocyte development, ErbB3 and ErbB4-specific ligands will also be of great importance for the determination of the individual roles of ErbB3 and ErbB4 in multiple sclerosis (37) and schizophrenia (38). Several genetic studies have identified NRG1β as a susceptibility gene for schizophrenia (39). Mice heterozygous for mutations in the NRG1β gene or with a null mutation in the ErbB4 gene display phenotypic characteristics similar to schizophrenia. Furthermore, significant reduction of ErbB3 expression has been observed in schizophrenia patients. NRG1 plays a key role in neuronal developmental processes in the central nervous system, including neuronal migration and specification, hormonal control of puberty, regulation of acetylcholine, GABA and glutamate receptor expression and oligodendrocyte development. (reviewed in (40)). However the exact role of the individual NRG receptors, ErbB3 and ErbB4, in these processes is not fully understood since NRG activates both receptors. Many NRG1-mediated processes have been found to be deregulated in schizophrenia patients, resulting in heterotopias, enlarged ventricles, alterations in neurotransmitter receptor expression, reduced myelination, reduced number of oligodendrocytes and disordered sexual maturation of the hypothlamus in the puberty. By administration of ErbB3 or ErbB4-specific ligands to NRG knockout mice, the contribution of the individual NRG receptors in the above-mentioned processes could be studied in more detail. ErbB specific ligands may therefore be of therapeutic value since they can activate one of the two neuregulin receptors without affecting the other.

9.7 Design of ErbB antagonists

The resolution of the crystal structure of unliganded and ligand-bound ErbB receptors has been a breakthrough in our understanding of ligand-mediated receptor dimerization. The crystal structures provide important information about possibilities to interfere with receptor dimerization, which could be of great therapeutic value. Here we will focus on the possible

development of receptor antagonists and reverse agonists. An antagonist competes effectively with ErbB agonists for binding to ErbB receptors but fails to induce domain rearrangement of the receptors which is required for ErbB signaling. Reverse agonists do not compete with agonistic ligands for receptor binding but prevent the receptor from adopting the conformation required for dimerisation. Additional crystal structures have identified the epitopes on ErbB receptors which are recognized by therapeutic antibodies, and have thereby attributed to our understanding of the working mechanism of these antibodies (41, 42). Several strategies have been used to interfere with ErbB signaling, of which the use of anti-receptor antibodies has so far been most successful. Cetuximab, a monoclonal antibody directed against ErbB1, interacts exclusively with domain III of ErbB1, thereby blocking the ligand binding region of this domain and preventing the ligand-induced domain rearrangement of the receptor (41) (Figure 7C). Blocking the dimerization interface is another option for preventing receptor activation. This has been shown for pertuzumab, a monoclonal antibody directed against ErbB2, that sterically blocks ErbB2-mediated dimerization (42) (Figure 7G). The most successful anti-ErbB therapy is treatment with Herceptin, an antibody directed against the S2 domain of ErbB2, which blocks a protease cleavage site in the receptor and thereby prevents the formation of a constitutively active p95 fragment, which is frequently found in breast tumors (Figure 7I).

Alternatively mutant forms of EGF-like growth factors with antagonist activity can potentially be designed, e.g. by mutating one of the receptor

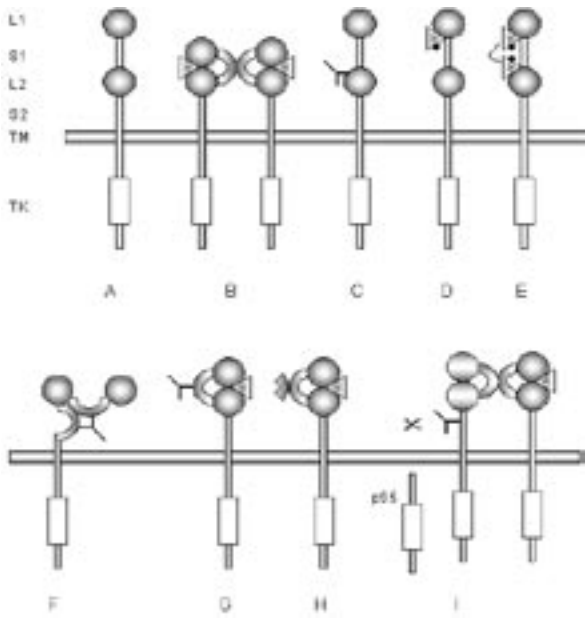

Figure 7. Strategies for the development of ErbB antagonists. (**A**) ErbB receptor in its extended conformation. (**B**) Ligand-bound ErbB dimer. (**C**) Cetuximab blocks one of the ligand binding domains. (**D**) ErbB antagonist interacting with one of the ligand binding domains whereby mutations prevent the interaction with the other ligand binding domain. (**E**) Two ErbB ligands with flexible linker. One of the ligands can only bind the L1 domain while the other can only bind the L2 domain. (**F**) Antibody that locks the ErbB receptor in its autoinhibited conformation. (**G**) Pertuzumab blocking the dimerisation interface. (**H**) S1 mimetic peptide blocking the dimerisation interface. (**I**) Herceptin preventing ectodomain cleavage of ErbB2.

binding domains in the ligand molecules, while leaving the other intact. This will initially result in a ligand with very low affinity, since half of the positive constraints for receptor binding will be lacking. It will be a challenge to improve the affinity of the remaining receptor binding domain by ligand extension and phage display approaches to design an antagonist with wild-type affinity. Alternatively, ligand-based reverse agonists can be designed by linking two ligand molecules with a spacer, such that after binding the L domains of the receptor are kept far apart from each other in order to prevent the receptor from adopting the conformation required for dimerisation (Figure 7B). This elongated ligand may exist of two EGF molecules that are separated by a flexible linker. The C-terminal EGF domain of this elongated ligand requires mutations in the C-loop and C-terminal linear region involved in L2 domain binding, whereas the L1 binding capacity of the N-terminal EGF domain has to be impaired by mutations in the B-loop (Figure 7E). As an alternative, mimetic peptides of the S2 domain have been made which were able to block receptor dimerization (43) (Figure 7H).

In the case of ErbB4, anti-receptor antibodies that prevent exodomain cleavage might be of therapeutic value. ErbB4 can be cleaved by TACE and γ-secretase at the extracellular juxtamembrane region and intramembraneous region, respectively. The intracellular domain (ICD) of ErbB4 translocates to the nucleus where it acts as a transcriptional cofactor. The cleaveable JM–a isoform is more abundantly expressed in breast cancers than the uncleavable JM-b isoform. Breast cancer patients with localization of an ErbB4 ICD epitope in the cancer cell nuclei have poor prognosis, compared with patients with cell surface ErbB4 expression (28). So by blocking ectodomain cleavage of ErbB4 receptors the formation of oncogenic or highly mitogenic receptor fragments can be prevented, similarly as is achieved by Herceptin for ErbB2.

An attractive alternative for the classical antibodies are camelid antibodies which consist only of a single heavy chain. The extended complementarity-determining region (CDR3) of camelid antibodies makes them very suitable for the recognition of epitopes that are normally not recognized by conventional antibodies. Because of their relatively small size, camelid antibodies have been selected for epitope recognition in enzymes based on the ability of CDR3 to penetrate into the substrate-binding pocket (44). The variable domains of these camelid antibodies, called nanobodies, have the advantage over the classical antibodies that they are very stable and easy to produce as recombinant protein. Because of their small size, they are easy to clone and expresses on filamentous phage which allows selection of specific high affinity binders to e.g. ErbB receptors. Using such phage display strategies antagonistic nanobodies have been selected with high affinity for ErbB1, which are able to prevent EGF-induced ErbB1 activation (45). Nanobodies lack an Fc-domain, and therefore they do not induce *antibody-dependent cellular* cytotoxicity (ADCC). For treatment of solid tumors with overexpression of ErbBs this does not have to be a problem since also pertuzumab is expected to give poor ADCC when blocking ligand

binding to ErbB1 (46). Due to their small size there is a rapid renal clearance of nanobodies. This has advantages for *in vivo* diagnosis studies or when targeting tumors with nanobody-coupled toxins. When treating tumors by blocking a biological process, e.g. ligand-receptor interaction, receptor dimerisation, or ectodomain shedding, however, such a short life span is a disadvantage. This can be overcome by the use of bispecific antibodies in which one nanobody recognizes albumin and the other the epitope of interest, which increases the lifetime of the nanobody. The potential of anti-ErbB directed nanobodies for treatment of cancer patients has to be further explored.

9.8 Possibilities for Argos-based design of antagonists for human ErbB receptors

Ever since the identification of Argos as an inhibitor of the Drosophila EGF receptor (DER) it has been proposed that Argos binds to DER without activating this receptor, thereby acting as a receptor antagonist (47). This hypothesis was based on the observation that Argos itself contains a single EGF-like domain (albeit a-typical). BIAcore measurements showed that Argos indeed binds with high affinity (17-41 nM) to DER (48). Several studies have shown that the EGF-like domain of Argos is essential and sufficient for its antagonistic activity (49). In particular, studies by Howes et al. (50) on Spitz-Argos chimeras showed that the B-loop and the C-terminal linear region are essential for the antagonistic activity of Argos. Other studies aimed to adapt Argos in such a way that it will inhibit human ErbB receptors, have been without success (18). However, in 2004 it has been reported that Argos inhibits DER signaling by sequestration of agonist ligands such as Spitz (51) and recently the crystal structure of Argos in complex with Spitz has been published (52). Although both mechanisms of antagonism (competitive binding to DER and ligand sequestration) are not mutually exclusive, there is currently more structural evidence for ligand sequestration, making this mechanism more plausible. Furthermore, crystal structure analysis has demonstrated that Argos does not have a similar folding as EGF, which makes it even more unlikely that Argos as a receptor antagonist by direct binding to DER.

The crystal structure of Argos revealed a striking similarity with the ligand binding regions of receptors for the TGFβ family of growth factors. Even more interestingly is the structural similarity with the cell surface receptor of urokinase-type plasminogen activator receptor (uPAR) which is known to interact with the EGF domain of uPAR. Since there are many proteins with a uPAR-like domain of unknown function, this group of proteins may contain a human orthologue of Argos. Even if no human uPAR orthologue with a three-finger toxin fold domains exists, Argos can potentially be used as a structural scaffold to design human scavengers of ErbB ligands.

Argos has been shown to interact with human EGF, although only with very low affinity (Kd ± 5 μM). This raises the question whether it is possible to optimize this interaction by mutating Argos. Among the 17 amino acids of Spitz that are directly involved in Argos binding, only 7 are conserved in human EGF (see Figure 8). Most interactions are formed by residues on the tip of the B-loop, which in the case of Spitz is two amino acids shorter than in human EGF. Furthermore the A-loop of Spitz is one amino acid longer than in EGF as a result of an additional tryptophan, which is clearly involved in a hydrophobic interaction. These differences in length of the A1-loop and the B-loop will make it very difficult to mutate Argos in such a way that the affinity for human EGF will be increased towards Kd values in the nanomolar level. Within the EGF-like domain there is relatively high amino acid sequence homology between Spitz and Keren. Based on this sequence homology we estimate that Keren would make 16 interactions with Argos compared to 20 interactions between Spitz and Argos (Figure 8). So in addition to Spitz, Keren might also be a candidate binding partner for Argos. Figure 8 also shows that the amino acids of Spitz which are involved in Argos binding are poorly conserved in Vein and Gurken, which makes it unlikely that DER activation by these ligands will be inhibited by Argos.

Figure 8. Interaction sites for binding of Spitz to DER and Argos. Presented sequences are of *Drosophila melanogaster* ligands Spitz, Keren, Gurken and Vein, in addition to human EGF, TGFA and NRG1β. The first amino acid and the conserved cysteines are numbered and are depicted in bold. The Spitz-Argos interactions are according Klein et al. (*52*). The Spitz-DER interactions are according to our homology modeling. The putative interactions of Keren, Gurken, Vein, EGF, TGFA and NRG1β are predicted on the basis of primary sequence homology and the chemical nature of the amino acid. Residues in red are involved in hydrogen bond formation, residues in green are involved in hydrophobic interactions, residues in blue are involved in salt bridge formation with the indicated ErbB receptors, while residues in pink are involved in both, hydrogen bound formation and hydrophobic interaction. Spitz is depicted twice, since some amino acids can be involved in both hydrogen bound formation and hydrophobic interactions.

Important lessons can be learned from the antagonistic mechanism of Argos, whereby it acts a scavenger for agonistic DER ligands. There are many examples in nature whereby soluble extracellular proteins act as growth factors scavengers. In comparison, there are only few examples of natural ligands that act as receptor antagonists, by binding a receptor without subsequent activation. Examples of ligands that act as receptor antagonists are inhibin (53) and LEFTY (54) for serine/threonine kinase receptors. Soluble extracellular regions of receptor molecules, either formed by alternative splicing or by protein shedding, can act as soluble ligand scavengers. Moreover, during evolution many unrelated proteins have developed that act as ligand scavengers (for instance WIF, Noggin and Gremlin). These scavengers play an important role in the formation of morphogen gradients which are essential for axis formation during development (55-57). Apparently during evolution the development of scavengers has appeared more effective than that of receptor-binding ligands with antagonist activity.

Argos is not the first protein with ErbB ligand scavenging activity. Recombinant extracellular domain fragments of ErbB1 have been shown to act as competitive inhibitors of EGF-stimulated mitogenesis (58). The full length extracellular domain, sEGFR621, has a relatively low affinity for EGF (Kd 180-300 nM) and TGFA (Kd 840-1320 nM), whereas the shorter sEGFR501 fragment has a much higher affinity for EGF (Kd 13-21 nM) and TGFA (Kd 35-40 nM). The sEGFR501 lacks the extracellular C2 domain and is therefore unable to adopt the auto-inhibited conformation and therefore has a higher affinity for EGF and TGFA (58). The full length soluble extracellular domain fragment of ErbB1 has been identified in the conditioned culture media of the A431 adenocarcinoma cell line and MDA-MB-468 breast cancer cells as a result of proteolytic shedding (59). It is unclear, however, whether this naturally occurring full length soluble extracellular domain fragment of ErbB1 indeed interacts *in vivo* with ErbB1 ligands in order to prevent them from activating membrane-bound ErbB1 receptors. Another natural ErbB antagonist is Herstatin, which is encoded by an alternative splice variant of the ErbB2 gene, and consists of the extracellular domain of ErbB2 followed by a proline rich region. Herstatin is not a scavenger of ErbB ligands but probably functions by blocking the major dimerisation interface (S1 or CR2 domain) of ErbB receptors and thereby prevents functional dimerisation. Furthermore Herstatin induces the downregulation of ErbB2 and inhibits NRG-induced proliferation of MCF7 and BT474 breast cancer cells (60).

A soluble p85 splice variant (61) of ErbB3 binds NRG1β with high affinity (Kd 3.0 nM) and thereby inhibits NRG-mediated activation of ErbB2, ErbB3 and ErbB4 (62). JM-a isoforms of ErbB4 can be cleaved by TACE and γ-secretase thereby releasing not only a transcriptionally active intracellular domain, but also the ligand binding extracellular domain of ErbB4. This extracellular domain of ErbB4 can function as a ligand to activate backward signaling via membrane-bound NRG1β isoforms (63). A functional role of the extracellular domain of ErbB4 in scavenging ligand has not been reported

so far. These naturally occurring ErbB ligand scavengers have similarly high affinity for their ligands as Argos for Spitz. Potentially such antagonists are interesting drugs for the treatment of carcinomas with show ErbB-mediated autocrine growth stimulation. The identification of uPAR or Argos-like three-finger toxin fold domains in human proteins which are able to antagonize ErbB signaling would therefore not only be interesting from a scientific point of view, but also opens possibilities for their use in anti-cancer therapies.

Remarkably all ErbB-directed strategies currently used in the clinic target the receptor. No strategies are aimed at scavenging the ErbB ligands with antibodies or nanobodies, while nature generally uses ligand scavengers to antagonize receptor signaling. Most probably therapies aimed at scavenging ErbB ligand are not an option for tumors which overexpress multiple ErbB ligands (64). Still, in the case of acquired tumor resistance to ErbB1 and ErbB2 directing drugs, scavenging NRG1β by a neutralizing antibody may be a promising approach, since siRNA targeting of NRG1β expression has been demonstrated to restore tumor sensitivity to ErbB1 and ErbB2 directing drugs (65).

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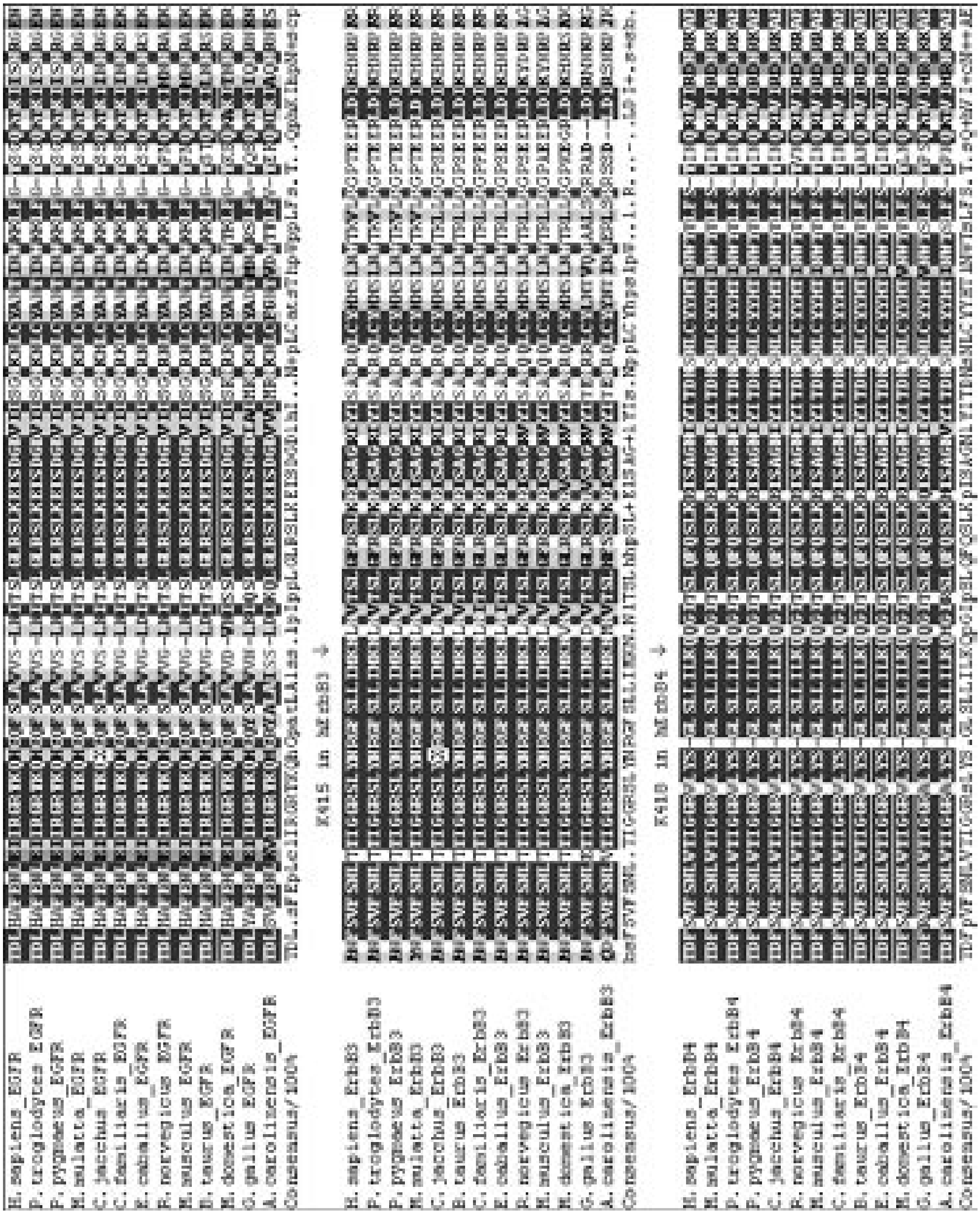
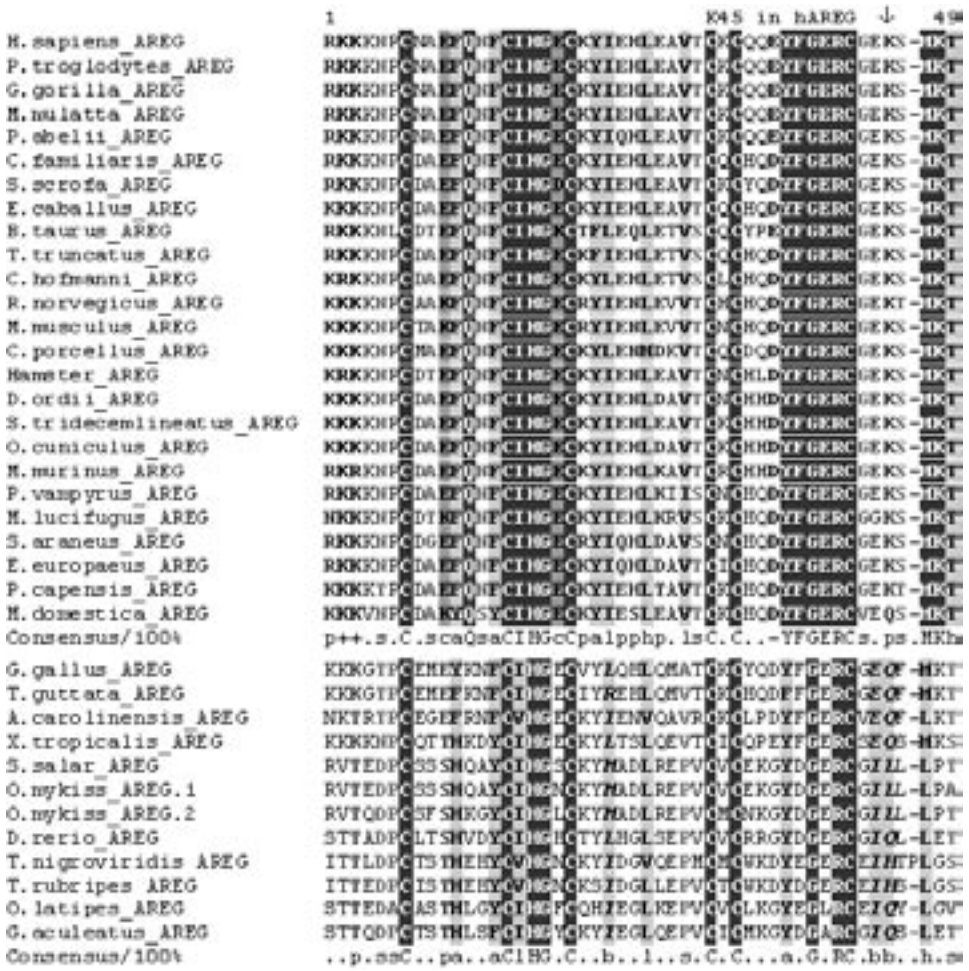
Figure 9. Sequence homology between tetrapod ErbB receptors (see next pages). Sequences of the extracellular domain of the ErbB receptors and the EGF-like domains of the ErbB ligands, were derived from Genbank files available as of 2009-03-01. Alignments were generated using ClustalW software, imported into the Chroma software package, and annotated using default black and white settings. ErbB1, ErbB3 and ErbB4 sequences were grouped into distinct sets and within each set, identity thresholds were set at 100% and consensus sequences were defined. Finally, the resulting alignment was exported into MS Word program. Sequences from *C. jacchus* (marmoset) were constructed from blast searches without the help of the underlying nucleotide sequence. The conservation of negative constraints in ligand and receptor are indicated with an arrow. Characteristics of the amino acids: Negative charge (-) DE; Positive charge (+) HKR; Aliphatic (l) ILV; Tiny (t) AGS; Aromatic (a) FHWY; Charged (c) DEHKR; Small (s) ACDGNPSTV; Polar (p) CDEHKNQRST; Hydrophobic (h) ACFGHILMTVWY and Big (b*)* EFHIKLMQRWY.

H.sapiens_EGFR
P.troglodytes_EGFR
P.pygmaeus_EGFR
M.mulatta_EGFR
C.jacchus_EGFR
C.familiaris_EGFR
E.caballus_EGFR
R.norvegicus_EGFR
M.musculus_EGFR
B.taurus_EGFR
M.domestica_EGFR
G.gallus_EGFR
A.carolinensis_EGFR
Consensus/100%
M97 in hErbB3
H.sapiens_ErbB3
P.troglodytes_ErbB3
P.pygmaeus_ErbB3
M.mulatta_ErbB3
C.jacchus_ErbB3
B.taurus_ErbB3
C.familiaris_ErbB3
E.caballus_ErbB3
R.norvegicus_ErbB3
M.musculus_ErbB3
M.domestica_ErbB3
G.gallus_ErbB3
A.carolinensis_ErbB3
Consensus/100%
H.sapiens_ErbB4
M.mulatta_ErbB4
P.troglodytes_ErbB4
P.pygmaeus_ErbB4
C.jacchus_ErbB4
R.norvegicus_ErbB4
M.musculus_ErbB4
C.familiaris_ErbB4
B.taurus_ErbB4
E.caballus_ErbB4
M.domestica_ErbB4
G.gallus_ErbB4
A.carolinensis_ErbB4
Consensus/100%

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K45 in hAREG
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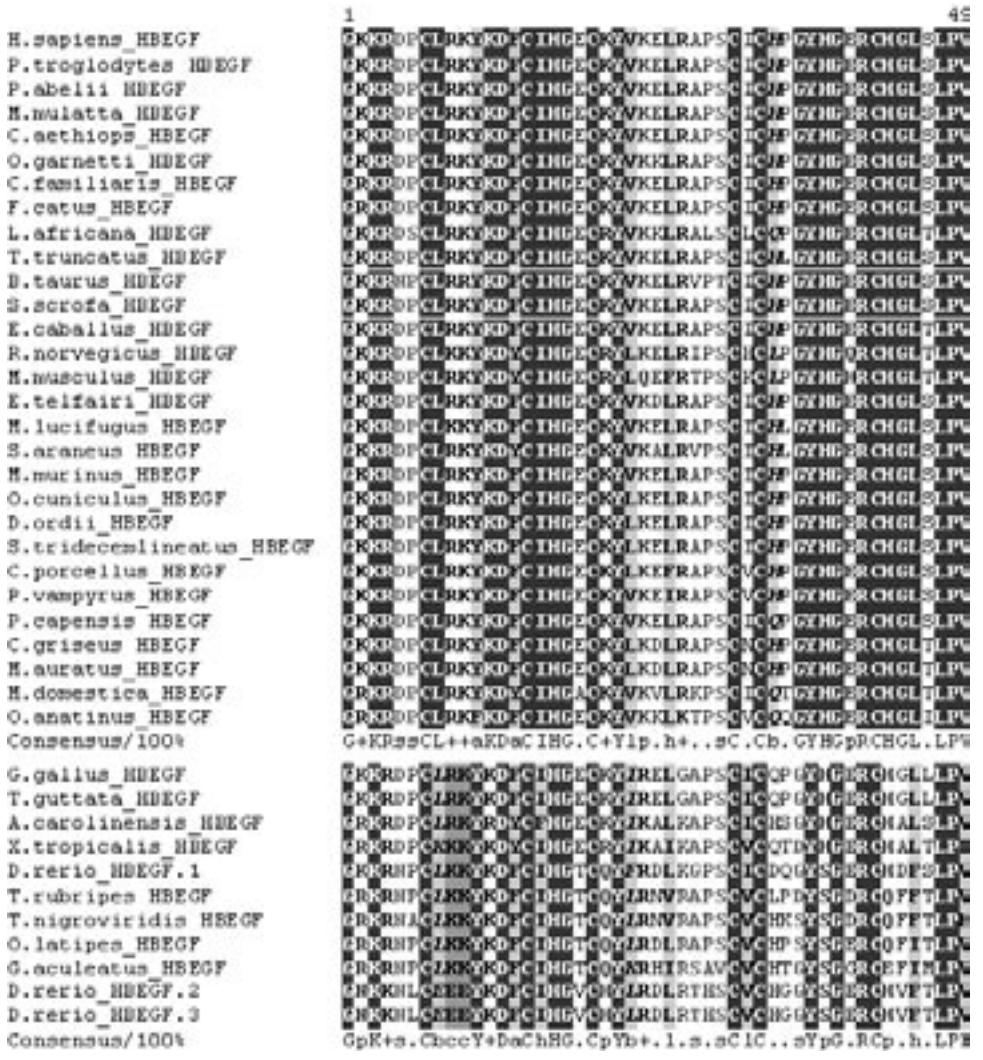
G.gorilla_AREG
M.mulatta_AREG
P.abelii_AREG
C.familiaris_AREG
S.scrofa_AREG
E.caballus_AREG
B.taurus_AREG
T.truncatus_AREG
C.hofmanni_AREG
R.norvegicus_AREG
M.musculus_AREG
C.porcellus_AREG
Hamster_AREG
D.ordii_AREG
S.tridecemlineatus_AREG
O.cuniculus_AREG
M.murinus_AREG
P.vampyrus_AREG
M.lucifugus_AREG
S.araneus_AREG
E.europaeus_AREG
P.capensis_AREG
M.domestica_AREG
Consensus/100%
G.gallus_AREG
T.guttata_AREG
A.carolinensis_AREG
X.tropicalis_AREG
S.salar_AREG
O.mykiss_AREG.1
O.mykiss_AREG.2
D.rerio_AREG
T.nigroviridis_AREG
T.rubripes_AREG
O.latipes_AREG
G.aculeatus_AREG
Consensus/100%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C.aethiops_HBEGF
O.garnetti_HBEGF
C.familiaris_HBEGF
F.catus_HBEGF
L.africana_HBEGF
T.truncatus_HBEGF
B.taurus_HBEGF
S.scrofa_HBEGF
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R.norvegicus_HBEGF
M.musculus_HBEGF
E.telfairi_HBEGF
M.lucifugus_HBEGF
S.araneus_HBEGF
M.murinus_HBEGF
O.cuniculus_HBEGF
D.ordii_HBEGF
S.tridecemlineatus_HBEGF
C.porcellus_HBEGF
P.vampyrus_HBEGF
P.capensis_HBEGF
C.griseus_HBEGF
M.auratus_HBEGF
M.domestica_HBEGF
O.anatinus_HBEGF
Consensus/100%
G.gallus_HBEGF
T.guttata_HBEGF
A.carolinensis_HBEGF
X.tropicalis_HBEGF
D.rerio_HBEGF.1
T.rubripes_HBEGF
T.nigroviridis_HBEGF
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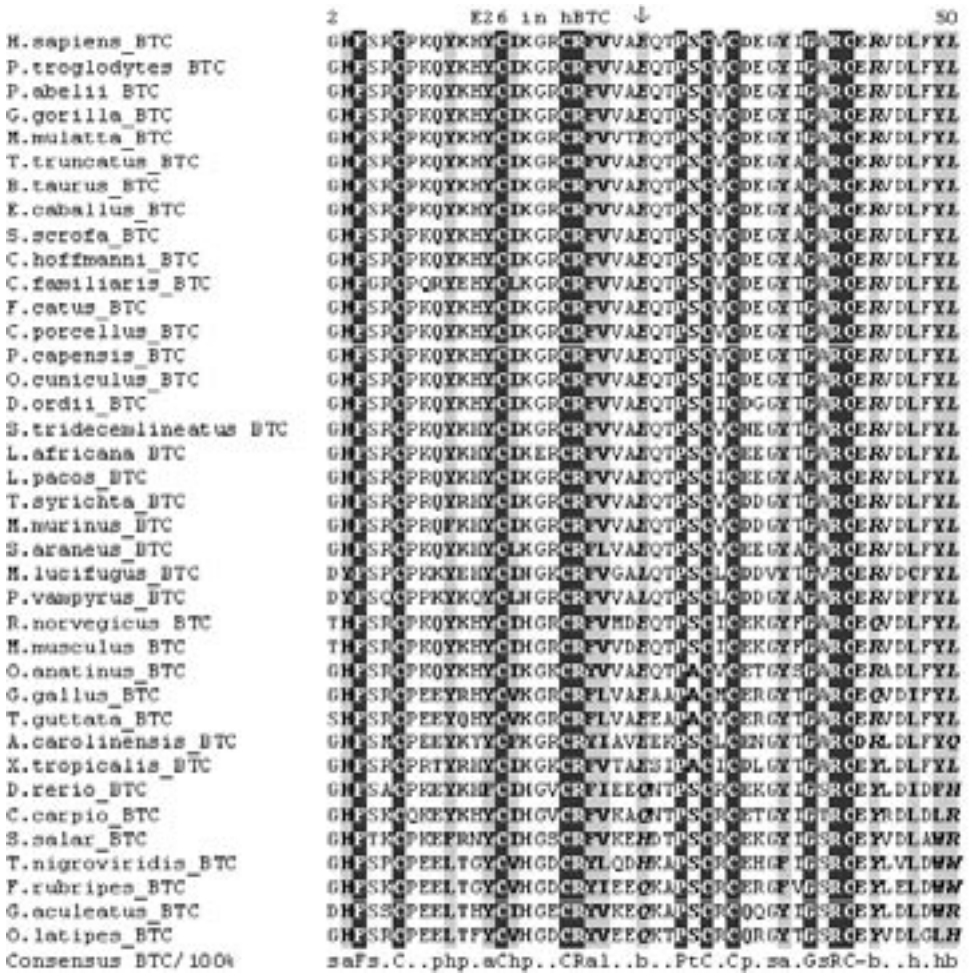
D.rerio_HBEGF.2
D.rerio_HBEGF.3
Consensus/100%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E26 in hBTC
50
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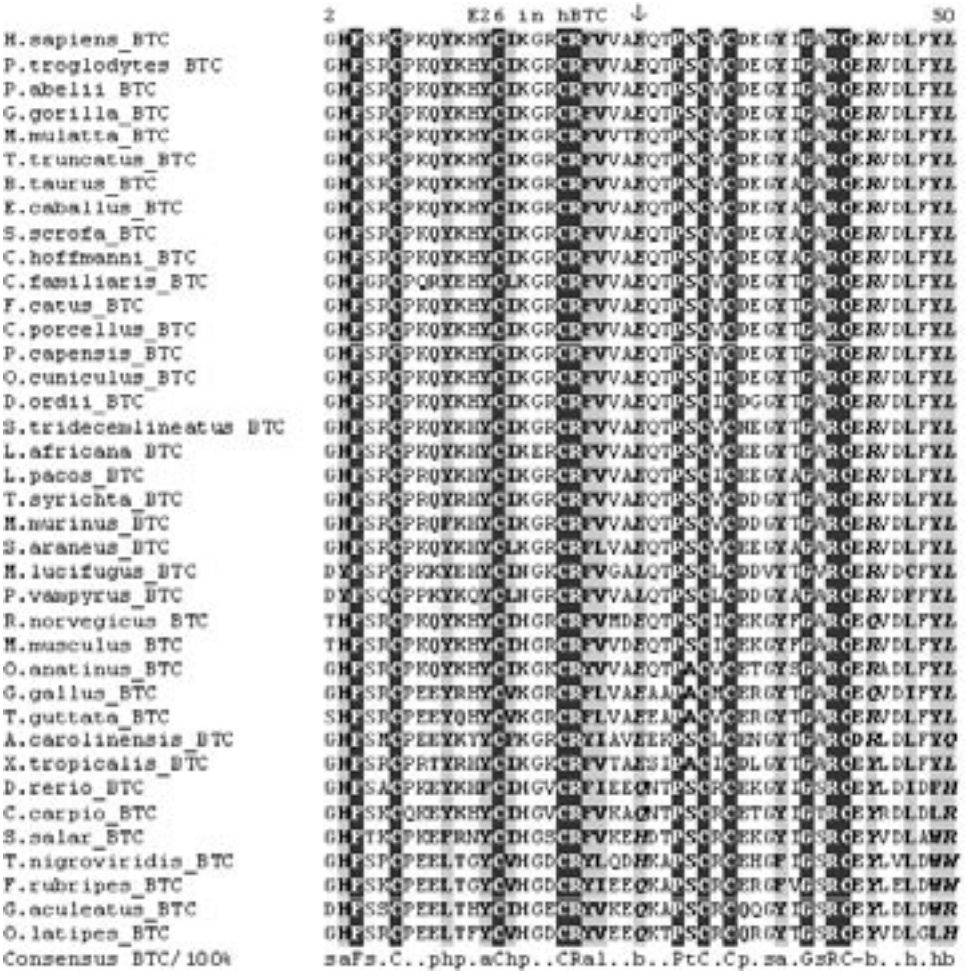
P.troglodytes_BTC
P.abelii_BTC
G.gorilla_BTC
M.mulatta_BTC
T.truncatus_BTC
B.taurus_BTC
E.caballus_BTC
S.scrofa_BTC
C.hoffmanni_BTC
C.familiaris_BTC
F.catus_BTC
C.porcellus_BTC
P.capensis_BTC
O.cuniculus_BTC
D.ordii_BTC
S.tridecemlineatus_BTC
L.africana_BTC
L.pacos_BTC
T.syrichta_BTC
M.murinus_BTC
S.araneus_BTC
M.lucifugus_BTC
P.vampyrus_BTC
R.norvegicus_BTC
M.musculus_BTC
O.anatinus_BTC
G.gallus_BTC
T.guttata_BTC
A.carolinensis_BTC
X.tropicalis_BTC
D.rerio_BTC
C.carpio_BTC
S.salar_BTC
T.nigroviridis_BTC
F.rubripes_BTC
G.aculeatus_BTC
O.latipes_BTC
Consensus BTC/100%


2
E26 in hBTC
50
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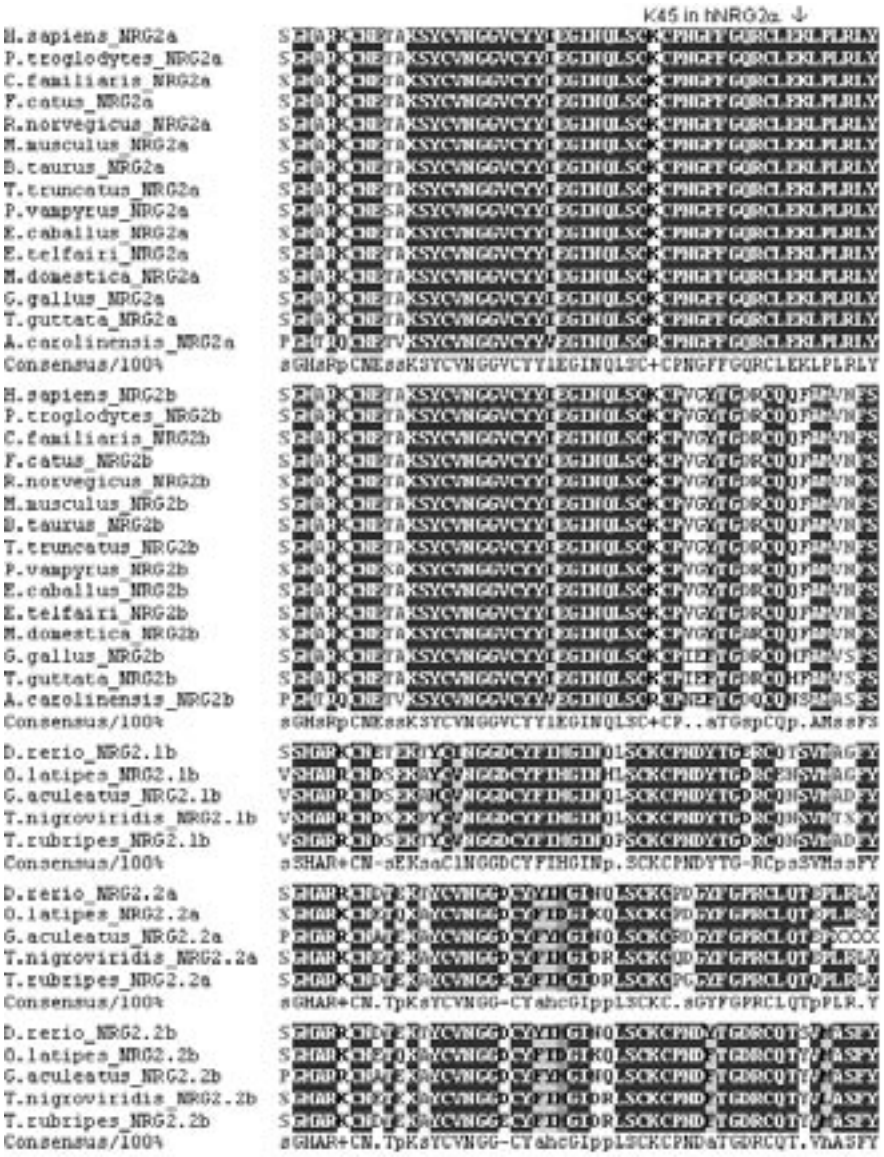
P.troglodytes_BTC
P.abelii_BTC
G.gorilla_BTC
M.mulatta_BTC
T.truncatus_BTC
B.taurus_BTC
E.caballus_BTC
S.scrofa_BTC
C.hoffmanni_BTC
C.familiaris_BTC
F.catus_BTC
C.porcellus_BTC
P.capensis_BTC
O.cuniculus_BTC
D.ordii_BTC
S.tridecemlineatus_BTC
L.africana_BTC
L.pacos_BTC
T.syrichta_BTC
M.murinus_BTC
S.araneus_BTC
M.lucifugus_BTC
P.vampyrus_BTC
R.norvegicus_BTC
M.musculus_BTC
O.anatinus_BTC
G.gallus_BTC
T.guttata_BTC
A.carolinensis_BTC
X.tropicalis_BTC
D.rerio_BTC
C.carpio_BTC
S.salar_BTC
T.nigroviridis_BTC
F.rubripes_BTC
G.aculeatus_BTC
O.latipes_BTC
Consensus BTC/100%


K45 in hNRG2α
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P.troglodytes_NRG2a
C.familiaris_NRG2a
F.catus_NRG2a
R.norvegicus_NRG2a
M.musculus_NRG2a
B.taurus_NRG2a
T.truncatus_NRG2a
P.vampyrus_NRG2a
E.caballus_NRG2a
E.telfairi_NRG2a
M.domestica_NRG2a
G.gallus_NRG2a
T.guttata_NRG2a
A.carolinensis_NRG2a
Consensus/100%
H.sapiens_NRG2b
P.troglodytes_NRG2b
C.familiaris_NRG2b
F.catus_NRG2b
R.norvegicus_NRG2b
M.musculus_NRG2b
B.taurus_NRG2b
T.truncatus_NRG2b
P.vampyrus_NRG2b
E.caballus_NRG2b
E.telfairi_NRG2b
M.domestica_NRG2b
G.gallus_NRG2b
T.guttata_NRG2b
A.carolinensis_NRG2b
Consensus/100%
D.rerio_NRG2.1b
O.latipes_NRG2.1b
G.aculeatus_NRG2.1b
T.nigroviridis_NRG2.1b
T.rubripes_NRG2.1b
Consensus/100%
D.rerio_NRG2.2a
O.latipes_NRG2.2a
G.aculeatus_NRG2.2a
T.nigroviridis_NRG2.2a
T.rubripes_NRG2.2a
Consensus/100%
D.rerio_NRG2.2b
O.latipes_NRG2.2b
G.aculeatus_NRG2.2b
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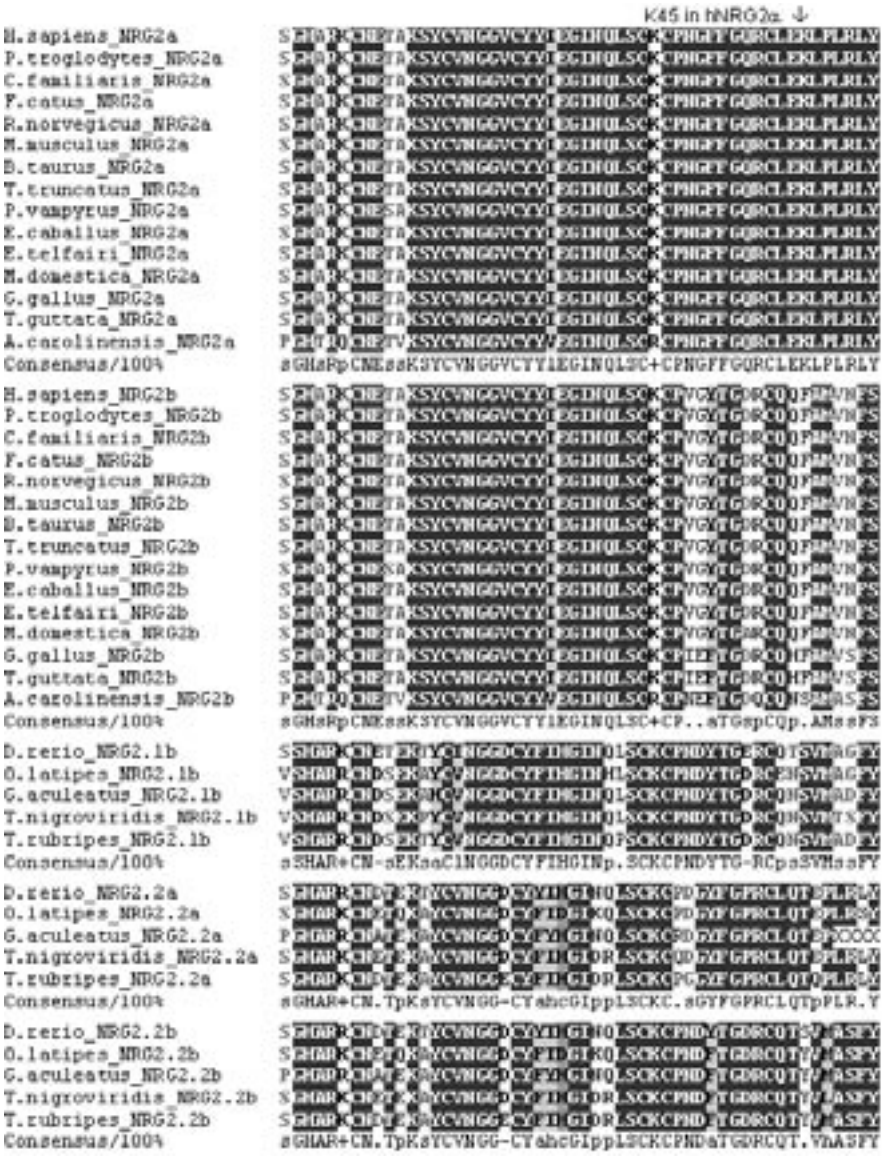
Consensus/100%


K45 in hNRG2α
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P.troglodytes_NRG2a
C.familiaris_NRG2a
F.catus_NRG2a
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E.telfairi_NRG2a
M.domestica_NRG2a
G.gallus_NRG2a
T.guttata_NRG2a
A.carolinensis_NRG2a
Consensus/100%
H.sapiens_NRG2b
P.troglodytes_NRG2b
C.familiaris_NRG2b
F.catus_NRG2b
R.norvegicus_NRG2b
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T.truncatus_NRG2b
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E.caballus_NRG2b
E.telfairi_NRG2b
M.domestica_NRG2b
G.gallus_NRG2b
T.guttata_NRG2b
A.carolinensis_NRG2b
Consensus/100%
D.rerio_NRG2.1b
O.latipes_NRG2.1b
G.aculeatus_NRG2.1b
T.nigroviridis_NRG2.1b
T.rubripes_NRG2.1b
Consensus/100%
D.rerio_NRG2.2a
O.latipes_NRG2.2a
G.aculeatus_NRG2.2a
T.nigroviridis_NRG2.2a
T.rubripes_NRG2.2a
Consensus/100%
D.rerio_NRG2.2b
O.latipes_NRG2.2b
G.aculeatus_NRG2.2b
T.nigroviridis_NRG2.2b
T.rubripes_NRG2.2b
Consensus/100%

Summary

SUMMARY

The ErbB family of receptor tyrosine kinases consists of ErbB1, ErbB2, ErbB3 and ErbB4, which all play a crucial role in the growth control of mammalian cells and the development of multicellular organisms. Depending on their ErbB binding specificity, EGF-like growth factors can be divided into three subfamilies: EGF, transforming growth factor-α (TGFα), amphiregulin (AREG) and epigen (EPGN) form a subfamily that binds exclusively to ErbB1, while the subfamily consisting of betacellulin (BTC), epiregulin (EREG) and heparin-binding EGF (HBEGF) binds not only ErbB1, but also ErbB4. Finally the neuregulins form a subfamily of which NRG1 and NRG2 bind both ErbB3 and ErbB4, while NRG3 and NRG4 appear specific for ErbB4. ErbB2 does not bind ligand by itself, but is the preferred heterodimerization partner for all liganded ErbBs and is particularly important for ErbB3 signaling which has a defective tyrosine kinase domain. The aim of this thesis is to understand the mechanisms of receptor specificity of the ErbB ligands.

In their mature form EGF-like growth factors contain approximately 55 amino acids and are characterized by three disulphide bridges which give rise to three looped structures and two terminal linear regions. The ligands bind to the L1 and L2 domain of the receptor, which causes these receptors to dimerize. Subsequently, the kinase domains of both receptors transphosphorylate the tyrosine residues in their intracellular tail. These phosphorylated tyrosine residues subsequently form docking sites for signaling molecules. Depending on the ErbB combination in the dimer and the cellular context, these intracellular signals can induce proliferation, migration or differentiation.

From crystal structure analysis of EGF or TGFα in complex with ErbB1, it is known that EGF-like growth factors contain approximately 20 residues that directly interact with their receptor. These residues, which we have referred to as positive constraints, form the basis for the observed high affinity receptor interaction. However, we have provided evidence that EGF-like growth factors contain in addition a limited number of residues that are not essential for high affinity binding to their cognate receptor, but are involved in ErbB selectivity. Using domain-exchange strategies, we have shown that mutation of Ser2/Asp3 within the N-terminal linear region of EGF into the corresponding residues His2/Phe3 from TGFα is sufficient to give EGF considerable affinity for ErbB3 and ErbB4. Similarly the point mutation E26L in the B-loop of TGFα has been shown to broaden its affinity to at least ErbB3.

Based on these changes in receptor selectivity by one or two amino acid mutations, we have postulated that EGF-like growth factors do not only contain residues that are directly involved in ErbB binding (positive constraints) but also residues that are not essential for binding to their cognate receptor

but prevent their unintended interaction with other ErbB receptors (negative constraints). The main aim in this thesis was to provide evidence that such negative constraints in ligand molecules and receptors form the primary basis for ErbB specificity.

In this thesis we have used a phage display approach to determine the requirements within the C-terminal linear region of ErbB ligands for optimal binding to different ErbB receptors. We have found that the ligand-binding receptors ErbB1, ErbB3 and ErbB4 have unique requirements for ligand binding, but that a consensus sequence can be derived that permits high affinity binding to all three receptors. From the data obtained, we concluded that the EGF/TGFα chimera T1E is a low affinity ligand for ErbB3 and ErbB4, because Arg45 forms a negative constraint for binding to ErbB3 and ErbB4 but not to ErbB1. Removal of this arginine from T1E resulted in the generation of a ligand, designated panerbin, that binds all three receptors with similarly high affinity as their wild-type ligands. Homology modeling of panerbin in complex with ErbB1, ErbB3 or ErbB4 showed that panerbin binds all three receptors in an almost identical manner, indicating that ErbB receptors recognize similar positive constraints in their ligand molecules. However, introduction of the identified negative constraints into panerbin resulted in local charge repulsion, conformational impairment, or steric hindrance for binding to ErbB3 and ErbB4 but not for ErbB1, thereby providing a molecular basis for their role in receptor specificity.

Introduction of the YYDLL sequence in naturally occurring ErbB ligands altered the receptor specificity of some EGF-like growth factors. The ErbB1-specific ligands EGF and TGFα broadened their receptor specificity towards ErbB4, without affecting their ErbB1 binding affinity. EREG, which by itself binds ErbB1 and ErbB4, but lacks affinity for ErbB3, broadened its binding specificity towards ErbB3. In case of NRG1β, introduction of the YYDLL sequence did not alter its receptor specificity but enhanced its ErbB3 binding affinity ten-fold. Although NRG/YYDLL showed a very high binding affinity to ErbB3, for unknown reasons its mitogenic potential towards ErbB2 and ErbB3 overexpressing cells was lower that that of wild-type NRG1β.

We have subsequently studied why NRG1β is unable to bind ErbB1. Comparison of the amino acid interactions of EGF and TGFα with ErbB1, and the possible interactions of NRG1β with ErbB3 and ErbB4 showed that the NRG1β-ErbB3/ErbB4 interaction is largely similar to EGF/TGFα-ErbB1 interaction. Domain-exchange studies between panerbin and NRG1β showed that the A1-loop and C-loop of NRG1β contain sequences that prevent ErbB1 interaction. It remains unclear, however, whether this implies that the A1 loop and C-loop of NRG1β lack ErbB1-specific positive constraints or that they contain negative constraints that prevent ErbB1 interaction. Remarkably, the A1-loop and the C-loop are oriented away from the receptor, making it unlikely that there are positive constraints for ErbB1 missing in the NRG1β A1-loop and C-loop or, alternatively, that these sequences contain negative constraints for ErbB1 interaction. How these NRG sequences are involved in the prevention of ErbB1 remains unclear, but we hypothesize that subtle

conformational changes in the loop structures that are not directly involved in receptor interaction, might alter receptor specificity.

The EGF receptor signaling network in *Drosophila melanogaster* consists of a single receptor (DER), which can be activated by four distinct ligands, designated Spitz, Keren Gurken and Vein. The Drosophila EGFR signaling network is unique in that it contains a soluble antagonist, Argos. Because in human epithelial tumors ErbB receptors are frequently overexpressed in combination with a continuous secretion of ligand, a humanized version of Argos would be very interesting to block the autocrine growth stimulation in these tumors. Therefore, we have investigated the compatibility between the human ErbB system and the DER system. DER overexpressing S2DER cells did not give a mitogenic response to human ErbB ligands and cells overexpressing human ErbB receptors did not respond to Spitz, Keren, Vein and Gurken. Although their overall structure is very similar, human and Drosophila EGF-like growth factors are unable to bind to each other's receptors. To determine why human EGF does not bind and activate DER and why *Drosophila* Spitz does not bind and activate human ErbB1, several hEGF-Spitz chimeras were designed. EGF in which the region between the third and the fifth cysteine had been replaced by Spitz sequences, bound with low affinity to both ErbB1 and DER, and was able to induce MAPK activation in both DER overexpressing S2DER cells and ErbB1 overexpressing HER14 cells. This indicates that the receptor specificity of Spitz and EGF does not result from structural differences between these ligands and their receptors. The lack of cross-reactivity between the EGF receptor signaling network of both species may reflect their evolutionary difference, whereby positive constraints and protein conformation have evolved differently in such a way that it does not permit binding to each other's receptor.

The artificial ErbB ligands we designed have been shown to be very useful in the determination of the ErbB number on breast cancer cells. Using panerbin as a radiolabeled ligand, we have been able to determine the ErbB expression profile of a number of ErbB overexpressing human breast tumor cell lines by a single radioreceptor assay. Using concentrations of [^{125}I]-panerbin that nearly saturate all ErbB receptors, we were able to determine by competition analysis with unlabeled BTC, NRG and TGFα the ratio of the number of ErbB1 + ErbB4, ErbB3 + ErbB4 and ErbB1 levels, respectively. The combination of the total number of panerbin binding sites with the ratio of the ErbB receptors allowed the determination of the number of all three ligand binding ErbB numbers on the cell surface, separately.

Determination of ErbB3 numbers on the cell surface of living cells using NRG1β is problematic. In our hands iodinated NRG1β is unstable and tends to underestimate the number of ErbB3 receptors in a radio-receptor assay. NRG/YYDLL is much more stable and using [^{125}I]-NRG/YYDLL as a label we observed 5-10 fold higher levels of ErbB3 receptors/cell than with [^{125}I]-NRG1β. Using near saturating concentrations of [^{125}I]-NRG/YYDLL a separate estimate of the total number of ErbB3 and ErbB4 receptors was made by

studying differential competition with unlabeled NRG/YYDLL and BTC.

All ErbB ligands show similar patterns of positive constraints that from a basis for binding to their receptor. Fine-tuning of these positive constraints can enhance the affinity by which these ligand bind their receptor. Receptor selectivity is regulated by negative constraints. In this thesis we used homology modeling to characterize three of those negative constraints. Ser2/Asp3 in the N-terminal linear region of EGF impaired ErbB3 and ErbB4 binding by causing an unstructured N-terminal linear region. Glu26 in TGFα caused a steric clash with Met97 in ErbB3 and Arg45 in EGF caused a charge repulsion with K415 in ErbB3 and K418 in ErbB4. Furthermore we observed that these negative constraints in receptor and ligand are highly conserved in vertebrate. So far we did not identify ErbB1-specific negative constraints in NRG1β, but it is reasonable to assume that they exist. Finally, we suggest that a combination of positive and negative constrains has put evolutionary pressure on the maintenance of multiple ErbB receptors and ligands.

SAMENVATTING

De ErbB familie van tyrosine kinases bestaat uit ErbB1, ErbB2, ErbB3 en ErbB4, die alle een belangrijke rol spelen bij de proliferatie van zoogdiercellen en de ontwikkeling van multicellulaire organismen. Afhankelijk van hun ErbB specificiteit zijn de EGF-achtige groeifactoren te verdelen in drie subfamilies: EGF, transforming growth factor-α (TGFα), amphiregulin (AREG) en epigen (EPGN) behoren tot een subfamilie die uitsluitend aan ErbB1 binden, terwijl de subfamilie bestaande uit betacellulin (BTC), epiregulin (EREG) en heparin-binding EGF (HBEGF) niet alleen aan ErbB1 bindt, maar ook aan ErbB4. Tenslotte vormen de neuregulins een subfamilie waarvan NRG1 en NRG2 zowel ErbB3 als ErbB4 binden, terwijl de bindingsspecificiteit van NRG3 en NRG4 nog beter gekarakteriseerd moet worden. ErbB2 bindt zelf geen ligand, maar is de preferentiële heterodimerisatiepartner voor de ligand-gebonden ErbBs. ErbB2 is vooral belangrijk voor ErbB3 signalering, omdat ErbB3 een defect tyrosine kinase domein heeft. Het doel van dit proefschrift is om de mechanismen die ten grondslag liggen aan receptorspecificiteit van ErbB liganden, te begrijpen.

In hun mature vorm bestaan EGF-achtige groeifactoren uit ongeveer 55 aminozuren en worden zij gekarakteriseerd door drie disulfidebruggen, die zorgen voor drie loop-structuren en twee terminale lineaire regio's. De liganden binden aan de zogenoemde L1 en L2 domeinen van de receptor, hetgeen resulteert in dimerisatie van de receptoren. Vervolgens fosforyleren de kinase domeinen van deze receptoren tyrosine-residuen in de intracellulaire staart van hun partner receptor. Deze gefosforyleerde tyrosine-residuen vormen vervolgens bindingsplaatsen voor signaleringsmoleculen. Afhankelijk van de ErbB combinatie in de dimeer en de cellulaire context, induceren deze intracellulaire signalen proliferatie, migratie en differentiatie van de betrokken cellen.

Via kristalstructuuranalyse van EGF of TGFα in complex met ErbB1 is bekend, dat EGF-achtige groeifactoren ongeveer 20 aminozuren bevatten die een directe interactie aangaan met de receptor. Deze residuen, die wij positive constraints zijn gaan noemen, vormen de basis voor de hoge affiniteitsbinding met de receptor. Daarnaast hebben wij bewijs gevonden voor het bestaan van een beperkt aantal aminozuren die niet essentieel zijn voor hoge affiniteitsbinding aan de receptor, maar juist betrokken zijn bij ErbB selectiviteit. Door gebruik te maken van domeinuitwisselingsstrategieën hebben wij laten zien dat mutatie van Ser2/Asp3 in de N-terminale lineaire regio van EGF naar de corresponderende aminozuren van TGFα, His2/Phe3, voldoende is om EGF aanzienlijke affiniteit voor ErbB3 en ErbB4 te geven. Op een vergelijkbare wijze heeft de puntmutatie E26L in TGFα als effect dat de receptorspecificiteit wordt verbreed naar tenminste ErbB3.

Op basis van deze veranderingen in receptorspecificiteit door slechts een enkele aminozuurmutatie hebben wij de hypothese opgesteld dat EGF-achtige groeifactoren niet alleen amonozuren bevatten die direct betrokken

zijn bij receptorbinding (positive constraints), maar ook aminozuren die niet essentieel zijn voor hoge affiniteitsbinding en juist essentieel zijn om onbedoelde interacties met andere ErbB receptoren te voorkomen (negative constraints). Het doel van dit proefschrift is om bewijs aan te dragen dat zulke negative constraints de basis vormen voor aanwezige ErbB specificiteit.

In dit proefschrift hebben wij gebruik gemaakt van de phage display techniek om te onderzoeken welke aminozuursequenties in de C-terminale lineaire regio nodig zijn, om het ligand optimaal aan de verschillende ErbB receptoren te laten binden. Wij hebben gevonden dat de ligand-bindende ErbB receptoren unieke aminozuursequenties nodig hebben voor binding aan hun receptoren, maar dat een consensus-sequentie bestaat die toestaat dat een ligand aan alle drie de receptoren met hoge affiniteit kan binden. Op basis van de verkregen gegevens concluderen wij dat de EGF/TGFα chimera T1E een lage-affiniteitsligand is voor ErbB3 en ErbB4, omdat Arg45 een negative constraint vormt voor ErbB3 en ErbB4, maar niet voor ErbB1. Verwijdering van deze arginine uit T1E resulteerde in de vorming van een ligand, panerbin genoemd, die met hoge affiniteit aan alle drie de receptoren bindt. Homology modeling van panerbin in complex met ErbB1, ErbB3 en ErbB4 liet zien dat panerbin alle drie de receptoren op een vergelijkbare manier bindt, hetgeen aantoont dat ErbB receptoren vergelijkbare positive constraints herkennen in hun ligand moleculen. Introductie van de geïdentificeerde negative constraints in panerbin resulteerde in een locale ladingsafstoting, verandering van de conformatie of sterische hindering met ErbB3 en ErbB4, maar niet met ErbB1, hetgeen een bewijs is voor hun rol in de moleculaire basis van ErbB specificiteit.

Introductie van de YYDLL sequentie in natuurlijk voorkomende ErbB liganden bleek de receptor-specificiteit van enkele EGF-achtige groeifactoren. De ErbB1 specifieke liganden EGF en TGFα verbreedden hun selectiviteit naar ErbB4, zonder verandering in hun affiniteit voor ErbB1. EREG, dat zelf ErbB1 en ErbB4 bindt, maar geen affiniteit heeft voor ErbB3, verbreedde zijn affiniteit naar ErbB3. In het geval van NRG1β gaf introductie van de YYDLL sequentie geen verandering in receptor-specificiteit, maar wel een tienvoudige verhoging van de affiniteit voor ErbB3. Alhoewel NRG/YYDLL een zeer hoge affiniteit heeft voor ErbB3 is, om onbekende reden, de mitogene activiteit op cellen die ErbB3 en ErbB2 tot overexpressie brengen, lager dan die van NRG1β.

Vervolgens hebben wij bestudeerd waarom NRG1β niet aan ErbB1 kan binden. De aminozuur interacties van EGF en TGFα met ErbB1 bleken erg vergelijkbaar te zijn met die tussen NRG1β en ErbB3/ErbB4. Domeinuitwisse lingsstudies tussen panerbin en NRG1β lieten zien dat de A1-loop en C-loop van NRG1β sequenties bevatten die de interactie met ErbB1 voorkomen. Het is echter nog onduidelijk of de A1-loop en C-loop van NRG1β belangrijke positive constraints missen of juist negative constraints bevatten die de interactie met ErbB1 voorkomen. Merkwaardig is dat de A1-loop en C-loop van NRG1β beide van de receptor afgekeerd liggen, hetgeen het onlogisch maakt dat deze domeinen ofwel positive constraints missen ofwel ErbB1 specifieke negative constraints bevatten. Hoe deze NRG1β sequenties de interactie met ErbB1

voorkomen blijft onduidelijk, maar mogelijk kunnen subtiele conformationele veranderingen in de loop structuren die niet direct betrokken zijn bij receptor binding, veranderingen in receptor specificiteit teweeg brengen.

Het EGF receptor signaleringsnetwerk in *Drosophila melanogaster* bestaat uit slechts een enkele receptor (DER), die geactiveerd kan worden door vier verschillende liganden, te weten Spitz, Keren, Gurken en Vein. Het EGF receptor netwerk in Drosophila is uniek omdat het een extracellulaire, niet-membraangebonden antagonist bevat, genaamd Argos. Omdat in humane epitheliale tumoren ErbB receptoren vaak tot overexpressie komen, in combinatie met continue secretie van activerend ligand, zou een gehumaniseerde versie van Argos erg interessant zijn om de autocriene groeistimulatie in deze tumoren te blokkeren. Daarom hebben wij onderzocht of er wederzijdse interactie bestaat tussen het humane en het Drosophila EGF receptor netwerk. DER-overexpresserende S2DER cellen vertoonden geen mitogene respons na stimulatie met humane ErbB liganden, terwijl cellen die humane ErbB receptoren tot overexpressie brengen, niet reageerden op Spitz, Keren, Gurken en Vein. Ook al is de overall structuur van de humane en de Drosophila EGF-achtige groeifactoren sterk vergelijkbaar, zij zijn kennelijk niet in staat om aan elkaars receptoren te binden. Om te onderzoeken waarom humaan EGF niet aan DER bindt en waarom Spitz niet aan ErbB1 bindt, hebben wij verschillende EGF-Spitz chimeren gemaakt. EGF waarin de regio tussen het derde en vijfde cysteine is vervangen door de overeenkomstige sequenties uit Spitz, bleek wel in staat om met lage affiniteit aan beide receptoren te binden en om MAPK te activeren in zowel DER overexpresserende S2DER cellen als ErbB1 overexpressererde HER14 cellen. Dit geeft aan dat de receptor-specificiteit van EGF en Spitz niet het resultaat is van structurele verschillen. Het feit dat er geen cross-reactiviteit is waargenomen tussen de EGF receptor netwerken van beide species komt waarschijnlijk omdat zij evolutionair zeer verschillend zijn, waarbij positive constraints en eiwit-conformatie op een andere manier geëvolueerd zijn, waardoor de liganden niet meer compatibel zijn met elkaars receptoren.

De artificiële ErbB liganden die wij hebben gemaakt, bleken zeer waardevol in het bepalen van de aantallen ErbB receptoren op borsttumorcellen. Door gebruik te maken van radioactief gelabeld panerbin waren wij in staat om het ErbB expressie-profiel van een aantal ErbB overexpresserende borsttumorcellen in weefselkweek te bepalen met behulp van een enkele radio-receptorassay. Door gebruik te maken van [^{125}I]panerbin bij een concentratie dat nagenoeg alle ErbB receptoren bezet zijn, hebben wij aan de hand van competitiecurves met BTC, NRG en TGFα, respectievelijk de ratio ErbB1 + ErbB4, ErbB3 + ErbB4 en ErbB1 bepaald. De combinatie van het totaal aantal panerbin bindingsplaatsen samen met de ratio van de ErbB receptoren maakte het mogelijk de aantallen van alle drie de ligand-bindende ErbB receptoren op het celoppervlak te bepalen.

Het bepalen van het aantal ErbB3 receptoren op het celoppervlak is problematisch. In onze handen is het gejodeerde NRG1β instabiel, waardoor het aantal bindingsplaatsen in een radio-receptor assay wordt onderschat. [^{125}I]NRG/YYDLL blijkt veel stabieler en gebruik makend van [^{125}I]NRG/YYDLL

als label vinden wij 5 tot 10 keer meer ErbB3 receptoren per cel dan met [^{125}I]NRG1β. Door gebruik te maken van bijna verzadigende [^{125}I]NRG/YYDLL concentraties hebben wij aldus het totaal aantal ErbB3 en ErbB4 receptoren kunnen bepalen. Vervolgens kon de ratio tussen ErbB3 en ErbB4 receptoren worden bepaald door competitiestudies met ongelabeld NRG/YYDLL en BTC.

Alle ErbB liganden laten een vergelijkbaar patroon van positive constraints zien, hetgeen de basis vormt voor receptor binding. Fine tuning van deze positive constraints kan de affiniteit verder verhogen, terwijl receptor specificiteit met name door negative constraints wordt gereguleerd. In dit proefschrift hebben wij homology modeling gebruikt om drie van dergelijke negative constraints te karakteriseren. Ser2/Asp3 in EGF voorkomt ErbB3 en ErbB4 binding vanwege een ongestructureerde N-terminale lineaire regio. Glu26 in TGFα veroorzaakt een sterische ontoegankelijkheid met Met97 in ErbB3, terwijl Arg45 in EGF een ladingsafstoting met K415 in ErbB3 in K418 in ErbB4 veroorzaakt. Verder hebben wij aangetoond dat de negative constraints in de ErbB liganden sterk geconserveerd zijn in vertebraten. Tot nu toe zijn er nog geen ErbB1-specifieke negative constraints geïdentificeerd in NRG1β, maar onze studies geven aanwijzingen dat deze bestaan. Uiteindelijk suggereren onze gegevens dat een combinatie van positive en negative constraints gedurende de evolutie druk heeft uitgeoefend heeft op het behouden van meerdere ErbB liganden en receptoren

DANKWOORD

Eindelijk is het dan zover! Al is het mij net niet gelukt om binnen 10 jaar te promoveren, toch ben ik trots op het geheel dat zo dadelijk naar de drukker gaat. Velen hebben mij in de afgelopen tijd gevraagd of ik er ooit aan heb gedacht te stoppen met dit promotieonderzoek. Nooit!

Op de eerste plaats wil ik mijn begeleider en promotor Joop van Zoelen bedanken voor de begeleiding van mijn onderzoek en voor zijn hulp bij het schrijven van artikelen en het manuscript. Joop, je bleef er in geloven dat het tot een goed einde zou komen. Naast de wetenschap is er bij jou ook ruimte voor ontspanning. Pot-luck bij jou thuis en de hele afdeling mee uit dineren en naar de musical, ik kon het allemaal erg waarderen.

De EGF-groep, te weten Monique van de Poll, Catelijne Stortelers, Miriam Wingens, Saskia Jacobs-Oomen en Robert Doornbos, wil ik bedanken voor praktische en theoretische inbreng van het onderzoek, maar ook voor een gezellige tijd. Verder wil ik met toenaam Walter van Rotterdam bedanken voor al het praktische werk dat hij voor mijn onderzoek heeft gedaan. Walter, je ervaring en nauwkeurigheid hebben een grote bijdrage geleverd bij het behalen van de resultaten die hebben geleid tot dit proefschrift.

Bart, bedankt voor al je hulp bij het wegwijs maken in InDesign.

Janny, bedankt voor het checken van de layout en voor je zorgzaamheid.

Ester Piek, bedankt voor de ruimte die je me gaf om mijn artikelen en mijn proefschrift af te ronden.

Ingrid, zonder jou was het voor mij niet mogelijk om mijn onderzoek bij Toegepaste Biologie te combineren met het afronden van mijn promotie. Bedankt daarvoor.

Ester, Leonie, Laura, Mascha, Sander, Suzanne en Bart, ik weet dat jullie als eerste op zoek gaan naar deze alinea, voor jullie de belangrijkste tekst in het hele proefschrift. Naast het praten over het wel en wee dat bij promoveren komt kijken, is er ook vriendschap ontstaan. We hebben er een bijzonder gezellige tijd van gemaakt. De borrels op het lab, de etentjes in de stad en de weekendjes weg. En fin, ik hoef het jullie niet allemaal uit te leggen. Ook al spreken we elkaar niet meer dagelijks, het is altijd gezellig als we elkaar weer zien en ik hoop dat dat nog heel erg lang zo kan blijven.

En dan Inez, Ingrid, Marlinda en Eugene… Ik wil jullie bedanken voor alle gezelligheid en dat jullie me bleven vragen voor borrels en het weekendje weg, terwijl ik meestal moest afzeggen. Nu ik vader ben geworden, druk

ben om mijn promotie af te ronden en een emigratie naar België aan het voorbereiden ben, kan ik niet meer zo vaak met jullie gezellige dingen doen, maar we houden contact.

Jurgen en Camiel, jullie frequente informeren naar de vorderingen in de 'fruitvliegelarij' stak een hart onder de riem;-)

Mijn ouders wil ik bedanken omdat ze mij altijd gestimuleerd hebben om te studeren en dat ook voor mij mogelijk hebben gemaakt. Papa, het is moeilijk voor mij te accepteren dat je er niet meer bent, zeker op de dag dat ik promoveer. Ik weet dat je er graag bij had willen zijn en dat je trots op mij bent.

Lieve Sam, als ik na een dag hard werken al mijn vensters op mijn computer sluit en je foto als achtergrondafbeelding te voorschijn komt, denk ik "snel naar huis, naar ons mooie kereltje".

Lieve Merel, bij alles wat ik doe steun je mij. Je bent voor mij van Amsterdam, waar de meeste van je vrienden wonen, naar Lent gekomen. En nu jij je contacten hier in Lent hebt opgebouwd, verhuizen we weer naar België en heb jij zelfs je baan opgegeven. Ik besef dat ik erg veel van je vraag. Ik ben erg blij met je en je maakt me heel gelukkig.

Op naar België!

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CURRICULUM VITAE

Sebastian Paul van der Woning werd geboren op 6 maart 1974 te Borne. Na het behalen van zijn MAVO diploma in 1990 en zijn HAVO diploma in 1992 begon hij aan de Hogere Laboratorium Opleiding te Enschede met een opleiding Medische Biochemie. Met een stage op de afdeling Celbiologie van de Faculteit Natuurwetenschappen, Wiskunde en Informatica van de toenmalige Katholieke Universiteit Nijmegen rondde hij zijn opleiding in de medisch biochemische richting af in 1996. Aansluitend studeerde hij Biologie aan de Katholieke Universiteit Nijmegen. Tijdens deze studie heeft hij stages gelopen op de afdeling Tumor Immunologie van de medische faculteit van de Katholieke Universiteit Nijmegen waar hij, onder begeleiding van dr. Franka Hartgers, werkte aan de karakterisatie van DC-STAMP, een eiwit dat specifiek in dendritische cellen tot expressie komt. Daaropvolgend liep hij een stage bij de afdeling Pathologie van de Universiteit van Maastricht, waar hij onder begeleiding van dr. Rene Hoet werkte aan phage display antilichaam-selecties tegen een nieuw transmembraan-eiwit dat eveneens specifiek tot expressie komt op dendritische cellen. Na het behalen van zijn doctoraal diploma in januari 2000 begon hij aan een promotie-onderzoek op de afdeling Celbiologie van de Faculteit Natuurwetenschappen, Wiskunde en Informatica van de Katholieke Universiteit Nijmegen op een project gefinancierd door NWO-Chemische Wetenschappen. Van januari 2006 tot en met december 2009 werkte hij vervolgens als post-doc op de afdeling Toegepaste Biologie van de Katholieke Universiteit Nijmegen op een project aan mesenchymale stamcellen, gefinancierd door Organon BV (Schering-Plough). Sinds januari 2010 is hij werkzaam als Scientist bij arGEN-X te Gent, België.