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The four members of the ErbB family of receptor tyrosine kinases are involved in a complex array of combinatorial interactions involving homo and heterodimers. Since most cell types express more than one member of the ErbB family, it is difficult to distinguish the biological activities of different homo and heterodimers. Here we describe a method for including homo or heterodimerization of ErbB receptors using synthetic ligands without interference from the endogenous receptors. ErbB receptor chimeras containing synthetic ligand binding domains (FKBP or FRB) were homodimerized using the bivalent FKBP ligand, AP1510, and heterodimerized using the bifunctional FKBP-FRB ligand, rapamycin. AP1510 treatment induced tyrosine phosphorylation of ErbB1 and ErbB2 homodimers and recruitment of SH2 domain containing proteins (Shc and Grb2). In addition, ErbB1 and ErbB2 homodimers activated downstream signaling pathways leading to Erk2 and Akt phosphorylation. However, only ErbB1 homodimers were internalized upon AP1510 stimulation and only ErbB1 homodimers were able to associate and induce phosphorylation of c-Cbl. Cells expressing AP1510-induced ErbB1 homodimers were able to form foci, however cells expressing ErbB2 homodimers displayed 5-7 fold higher focus forming ability. Using rapamycin-inducible heterodimerization we show that c-Cbl is unable to associate with ErbB1 in a ErbB1/ErbB2 heterodimer most likely because ErbB2 is unable to phosphorylate the c-Cbl binding site on ErbB1. Thus, we demonstrate that ErbB1 and ErbB2 homodimers differ in their ability to transform fibroblasts and provide evidence for differential signaling by ErbB homodimers and heterodimers. These observations also validate the use of synthetic ligands to study signaling and biological specificity of selected ErbB dimers in any cell type.

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FOREWORD

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(Senthil Muthuswamy)

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The proposed plan was to construct ErbB chimeras as myristylation domain fusion and use such chimera for synthetic ligand inducible dimerization. However, myristylation domain fused ErbB chimeras fail to express themselves in Rat1 fibroblasts. I'm not certain as to why these chimeras failed to express in Rat1 based cell lines, one possible explanation might be that the cells were not able to tolerate stable expression of EGFR kinase domain fused to the myrisylation sequence. Hence, the chimeric receptors have been redesigned as follows: the extracellular domain of ErbB receptors were deleted and replaced with the extracellular domain of the low affinity NGF receptor (p75) (Figure 1 in the enclosed manuscript). The small molecule drug binding domains were fused to the C-terminal tail of the chimera as described Figure 1 (in the attached manuscript). The cytoplasmic domains of ErbB1, ErbB2 and ErbB4 have been subcloned in to the expression vector to make the p75.B1; p75.B2; p75.B4 chimeric proteins (Technical objective #1, Year 1).

Stable cell lines expressing ErbB1 and ErbB2 chimeras have been generated using Rat1 fibroblasts (Figure 2, and 4). These cell lines were used to evaluate the biological and biochemical outcomes synthetic ligand induced receptor dimerization (Year 1 and part of Year 2). In the enclosed manuscript I have demonstrated that the synthetic ligand induced homodimers are able to undergo:

(1)	a dose-dependent tyrosine phosphorylation (Figure2, 4)
(2)	associate with cytoplasmic signaling protein in an activation dependent manner (Figure 3)
(3)	activation of downstream signaling molecules MAPK and Akt (Figure 2 and 4)
(4)	synthetic ligand induced dimers retain their kinase specificity- Previous studies have shown that ErbB1 homodimers can induce phosphorylation of c-Cbl whereas ErbB2 homodimers are unable to induce phosphorylation. Using the dimerization strategy I have developed I was able to show that synthetic ligand induced ErbB1 and ErbB2 dimers retain their kinase specific functions (Figure. 11)
(5)	like natural ligand activated ErbB1 receptors, synthetic ligand induced ErbB1 dimers also undergo activation dependent receptor internalization (Figure 5)
(6)	both ErbB1 and ErbB2 receptor activation using synthetic ligands results in stimulation of cell cycle progression (Figure 6)
(7)	Activation of receptor dimerization results in acquisition of transformed morphology (Figure 8)

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(8)   These studies provide first clear evidence that ErbB1 homodimers and ErbB2 homodimers a differ in their transforming abilities (Figure 7, and Table 1)

(9)   I have also demonstrated that cell lines expressing different chimeras can be established and homodimers or heterodimers can be selectively induced using homodimerizing or heterodimerizing synthetic ligands (Figure 9).

(10)   This approach reveals, for the first time, that ErbB1 homodimers and ErbB1/ErbB2 heterodimers may form different signaling complexes.

In conclusion, using Rat1 fibroblasts I have provided biochemical and biological evidence that strongly argues that small molecule induced dimerization strategy can be used to study homo and heterodimerization between ErbB family members (SOW for Year 1 and part of Year 2).

Abstract

The four members of the ErbB family of receptor tyrosine kinases are involved in a complex array of combinatorial interactions involving homo and heterodimers. Since most cell types express more than one member of the ErbB family, it is difficult to distinguish the biological activities of different homo and heterodimers. Here we describe a method for inducing homo or heterodimerization of ErbB receptors using synthetic ligands without interference from the endogenous receptors. ErbB receptor chimeras containing synthetic ligand binding domains (FKBP or FRB) were homodimerized using the bivalent FKBP ligand, AP1510, and heterodimerized using the bifunctional FKBP-FRB ligand, rapamycin. AP1510 treatment induced tyrosine phosphorylation of ErbB1 and ErbB2 homodimers and recruitment of SH2 domain containing proteins (Shc and Grb2). In addition, ErbB1 and ErbB2 homodimers activated downstream signaling pathways leading to Erk2 and Akt phosphorylation. However, only ErbB1 homodimers were internalized upon AP1510 stimulation and only ErbB1 homodimers were able to associate and induce phosphorylation of c-Cbl. Cells expressing AP1510-induced ErbB1 homodimers were able to form foci, however cells expressing ErbB2 homodimers displayed 5-7 fold higher focus forming ability. Using rapamycin-inducible heterodimerization we show that c-Cbl is unable to associate with ErbB1 in a ErbB1/ErbB2 heterodimer most likely because ErbB2 is unable to phosphorylate the c-Cbl binding site on ErbB1. Thus, we demonstrate that ErbB1 and ErbB2 homodimers differ in their ability to transform fibroblasts and provide evidence for differential signaling by ErbB homodimers and heterodimers. These observations also validate the use of synthetic ligands to study signaling and biological specificity of selected ErbB dimers in any cell type.

Introduction

ErbB family receptor tyrosine kinases, ErbB1 (also known as HER1 or EGFR), ErbB2 (also known as HER2 or Neu), ErbB3 and ErbB4, consist of an extracellular ligand-binding domain, a single transmembrane domain, an uninterrupted tyrosine kinase domain and a cytoplasmic tail. The ErbB family members play important roles during growth and development of a number of organs including the heart (9, 17), the mammary gland (9, 27, 77), and the central nervous system (9, 17, 28). In addition, ErbB overexpression is associated with tumorigenesis of the breast, ovaries, brain and prostate gland (1, 9, 36). Experiments in transgenic mice and cell culture models clearly indicate that ErbB receptors and their ligands can promote development and progression of mammary tumorigenesis (36, 74).

There are at least 16 different EGF family ligands that bind ErbB receptors (55). The ligands can be grouped into three categories: (1) those that bind to ErbB1 alone (EGF, TGFα, amphiregulin); (2) those that bind to ErbB3 and ErbB4 (neuregulin 1 (NRG1) and NRG2) and (3) those that bind to ErbB1 and ErbB4 (betacellulin, heparin binding-EGF, NRG3 and epiregulin) (32, 55). Binding of EGF family ligand to its cognate receptor results in dimerization and activation of the receptor (76).

ErbB family members partake in a complex process of lateral signaling (also referred to as combinatorial interactions) by forming ligand-induced heterodimers between different family members (1, 18, 55). It is likely that heterodimerization is mediated by ligand bivalency (71). Each ligand has been shown to favor certain dimeric combinations over others suggesting that in cells expressing all four ErbB receptors a given ligand induces a hierarchical order of ErbB receptor dimerization (72). Among the ErbB RTKs, ErbB2-containing heterodimers are preferred over other ErbB homo or heterodimers suggesting that ErbB2 plays a central role in both ligand binding and signal transduction (4, 29, 37, 52, 62, 73). EGF-stimulation of cells engineered to lack surface expression of ErbB1 results in defective ErbB2 phosphorylation, and EGF or NRG1-stimulation of cells engineered to lacksurface expression of ErbB2 results

in impaired ErbB1, ErbB3 and ErbB4 phosphorylation (30). Taken together, these observations highlight the importance of combinatorial interactions in ErbB receptor signaling. Although ErbB2 is recruited into many heterodimers it is likely that different heterodimers have distinct signaling specificities. The evidence that different ligands induce distinct phosphorylation patterns on ErbB1 and ErbB2 is consistent with this possibility (19a, 51).

Activation of ErbB receptors results in generation of Src Homology 2 (SH2) binding sites for multiple cytoplasmic signaling molecules such as the p85 subunit of phosphoinositide 3' kinase (PI 3'-kinase) (61), PLCγ_1 (16), Src family kinases (5), protein tyrosine phosphatases, SHP1 and SHP2 (25), Shc and Grb2 (13), Grb7 (20), Grb10 (20), c-Cbl (44) (47), Nck (6), Crk (6), Eps8 (22), and Eps15 (23). ErbB receptors also induce tyrosine phosphorylation of proteins involved in cell adhesion signaling such as the focal adhesion kinase (FAK) (54), Crk associated substrate (Cas) (50), paxillin (58), cortactin (8), and catenins (35). It is likely that different ErbB dimers recruit/activate different sets of signaling molecules. For example, the p85 subunit of PI3' kinase is thought to associate only with ErbB3 (24, 39, 53, 64), c-Cbl with ErbB1 (41), and Chk with ErbB2 (80). c-Src associates with both ErbB1 and ErbB2, though it appears that c-Src prefers ErbB2 over ErbB1 (49). Very little is known about how ErbB homodimers and heterodimers differ in their biological properties and it is also not known whether heterodimers possess unique signaling properties compared to homodimers. Since almost all fibroblasts and mammary epithelial cells express more than one member of the ErbB receptor family, it is not possible to determine the signaling and biological specificities of different ErbB receptor homo or heterodimers using natural peptide ligands. In this report, we demonstrate that synthetic dimerizing ligands can be used effectively to study homo and heterodimerization of chimeric ErbB receptors independent of endogenous receptors and their ligands.

Synthetic dimerizing ligands have been used to induce dimerization and activation of transcription factors (33, 57), T cell receptor subunits (70), Src family kinases (69), the guanine nucleotide exchange factor, SOS, (34), PDGF receptor (78), caspases (45), Fas

receptor (68), erythropoetin receptor (7) and integrins (31). Here we demonstrate that synthetic ligand induced homodimerization of either ErbB1 or ErbB2 in rat fibroblasts results in tyrosine phosphorylation of the receptor, phosphorylation of downstream signaling molecules in a kinase-specific manner, induction of DNA synthesis, ligand-dependent foci formation and ligand-dependent acquisition of transformed morphology. Our results also indicate that ErbB1 homodimers were 5-7 fold weaker in their ability to induce focus formation compared to ErbB2 homodimers. In addition, using a synthetic ligand that selectively induce heterodimers, we demonstrate that c-Cbl prefers ErbB1 in a homodimer over ErbB1 in a heterodimer with ErbB2 suggesting that homo and heterodimers recruit distinct cytoplasmic signaling proteins.

Materials and Methods

DNA constructs

The expression vectors for the ErbB chimeras were constructed as follows: the extracellular and transmembrane domains of low affinity NGF receptor (p75) was PCR amplified as an EcoR1/BamH1 fragment and subcloned into the retroviral expression vector SRαMSVTKNeo (kindly provided by O.Witte). To generate the EcoR1/BamH1 fragment the 5' primer was engineered to have an EcoR1 site and the 3' primer was engineered to have inframe Spe1 and Xba1 sites followed by either a HA or Flag epitope tag, stop codons and BamH1 restriction site. The resulting vectors were referred to as either p75.HA or p75.Flag. The ligand binding domains (FKBP (one or two copies) and FRB (57) were subcloned as Xba1/Spe1 fragments into p75.HA and p75.Flag to generate p75.F1.HA (F1: one copy of FKBP) or p75.F2.HA (F2: two copies of FKBP) or p75.R1.Flag (R1: one copy of FRB domain) (Fig. 1B). FRB is the FKBP-Rapamycin Binding domain of FRAP (57). The intracellular domains of ErbB1 (B1) and ErbB2 (B2) were obtained by PCR using Pfu DNA polymerase (Stratagene). The primers were designed such that they contain inframe Xba1 and Spe1 restriction sites in the 5' and 3' end respectively. ErbB1 was amplified with the primers 5' GCGATCTCTAGACGAAGGCGGCCA CATCGTTCGG and 5' GCATCGACTAGTTGCTCCAATAAATTCACTGCTTTG using a T47D cDNA library (generated by random priming poly A selected mRNA). The kinase dead ErbB1 (kdB1) was amplified using the Met721Ala mutant human ErbB1 cDNA (kindly provided by Alan Wells). Both ErbB1 and kdB1 PCR fragments were subcloned into a shuttle vector digested with Xba1 and Spe1 restriction enzymes. The ErbB2 cytoplasmic domain was amplified as two fragments from a Rat Neu cDNA (kindly provided by William J. Muller) making use of an internal unique Nco1 site. The 5' fragment was amplified using 5'GCGATCTCTAGAAAACGAAGGAGACAGAAGATCC and

5'GGAGGTCGGGGTACCTGTCATGG primers. The 3' fragment was amplified using 5'CCATCCAGCCCCATGGACAGTACC and 5'GCATCGACTAGTTACAGGTACATCCAGGCCTAGG primers. The 5' and 3' fragments were subcloned into a Xba1/Spe1 cut shuttle vector by a three way ligation. The ErbB1 and ErbB2 PCR products, in shuttle vectors, were subject to automated sequencing to verify the nucleotide sequence. The intracellular domains of ErbB1, ErbB2 and kdErbB1 were subcloned as Xba1/Spe1 fragments into the Xba1 site in p75 fusion vectors (Fig.1B) to generate p75.B1.F1.HA, p75.B1.F2.HA, p75.B2.F2.HA and p75.kdB1.R1.Flag.

Retroviral stocks

Retroviral stocks were prepared using the phoenix packaging cells following the protocol outlined at *http://www.stanford.edu/group/nolan/NL-reteropage.html*. The viral stocks were stored at -80°C.

Cell culture and stable cell lines

Rat1 cells (kindly provided by Peter Siegel and William J. Muller) were grown in DMEM supplemented with 10% FBS and antibiotics. Stable cell lines expressing p75.B1.F1.HA, p75.B1.F2.HA and p75.B2.F2.HA were derived by infecting Rat1 fibroblasts with retrovirus expressing respective RTK chimera and selecting infected cells with 500 μg/ml G418 containing media. Clones were screened by either anti-HA blots or by florescence activated cell sorting (FACS) of cells stained with anti-p75 antibodies and FITC conjugated anti-mouse secondary antibodies . Clones that showed comparable levels p75 surface staining were used for the experiments (p75.B1.F1.HA clone-9, p75.B1.F2.HA clone 3 or 6, and p75.B2.F2.HA clone 4). Cells coexpressing FKBP and FRB (p75.kd.B1.R1.Flag) fusion were derived by transfecting the p75.B1.F2 (clone-6) and p75.B2.F2 (clone-4) expressing cells with the p75.kdB1.R1.Flag and pBabe Hygro (48) and selected in media

containing 200 μg/ml hygromycin (Boehringer Manheim). Hygromycin resistant colonies were pooled and early passages (between 3 and 10) were used for heterodimerization experiments (Fig.9). Transient assays in COS7 cells (Fig. 10) were carried out by plating 1.2 x10^{6} cells per 10 cm plate and lipofection was carried out 16-18 hrs after plating. The lipofection mix was prepared using 30 μl of lipofectamine; 3.0 μg of p75.kdB1.R1.Flag and 1.0 μg of either p75.B1.F2.HA or p75.B2.F2.HA following manufacturer's protocol (Gibco-BRL). Lipofection was carried out for 5 hrs and the cells were analyzed 48 hrs after transfection.

Cell lysis and immunoprecipitation.

Sub-confluent or confluent cultures were stimulated with indicated amounts of AP1510, or rapamycin for 15 minutes at 37°C. The ligands were stored as 2000x stock in 100% ethanol at -20°C. After stimulation, the cells were rinsed once with ice-cold PBS containing 1 mM Sodium orthovananadate and lysed in Triton X-100 lysis buffer (150 mM NaCl; 50 mM Tris.Cl (pH 8.0); 5 mM NaF; 1% Triton X-100; 1 mM Sodium orthovanadate; 5 μg/ml Aprotinin; and 5 μg/ml Leupeptin) for 25-35 minutes. The lysates were cleared by centrifugation at 13,000 rpm for 15 minutes at 4°C. Protein concentrations were measured using Bradford assay (BioRad). Cell lysates were incubated with anti-HA (HA.11, BabCo) or anti-flag (anti-Flag M2 beads, Sigma) or anti-Cbl (SC#14, Santacruz) antibodies in 500 μl total volume. Protein G Sepharose beads (Pharmacia) were added to the lysate-antibody mix and incubated on a rotating platform for a 2.5-3.5 hrs at 4°C and washed 3-4 times with lysis buffer. The immunoprecipitates or total cell lysates were resolved on a 8.0 or 9.0% SDS-PAGE gel and transferred onto PVDF membranes (NEN). The blots were blocked for 1.5-3.0 hrs in 3% BSA in TBS-T (20 mM Tris.Cl (pH 7.5); 150 mM NaCl; 0.1% Tween-20) and immunoblotted for 1.5-3.0 hrs with either anti-pTyr (PY20-HRP,

Transduction Labs, 1:3000) or anti-EGFR (1:1000, Transduction Labs) or, anti-Erk2 (1:1000, UBI), or antiphospho-473 Akt (1:1000, New England Biolabs), or anti-Akt (1:1000, New England Biolabs), or anti-Flag (1:1000, M2, BabCo,), or anti-Shc (1:1000, Transduction Labs) or anti-Grb2 (1:1000, Transduction Labs) or anti-beta1 integrin (1:1000) antibodies. The immunoblots were washed 5-7 and incubated with appropriate HRP conjugated secondary antibody subsequently, washed 5-7 times, reacted with Enhanced Chemiluminescence (NEN) and subject to autoradiography. To strip the blots were incubated in strip buffer (62.5 mM Tris.Cl (pH 6.8); 2% SDS; 0.7% mercaptoethanol) at 50°C for 30 min.

Receptor internalization experiments were carried out as follows: Cells were stimulated with Ap1510 or the ethanol alone for indicated lengths of time. The cells were rinsed three times with ice-cold PBS (pH 8.0) and incubated with PBS containing 0.5mg/ml NHS-S-S-Biotin (Pierce) at 4°C for 1 hour. The cells are subsequently rinsed three times with ice-cold PBS and were lysed in 1X modified radioimmunoprecipitation buffer (RIPA:150mM NaCl, 20mM Tris.HCL (pH 7.5), 0.1% SDS, 1.0% Sodium deoxycholate, 1.0% TritonX-100, $2\mu g/ml$ Aprotinin and $2\mu g/ml$ Leupeptin). Equal amounts of lysates ($300\mu g$) were incubated with $75\mu l$ of sepharose beads coupled to NeutrAvidin (Pierce) on a rotating platform for 1-1.5 hours. The immunoprecipitates were washed three times with modified RIPA and resuspended in 1X sample buffer.

Cell cycle analysis.

Parental Rat1 cells or p75.B1.F1.HA (clone-9) or p75.B1.F2.HA (clone-6) or p75.B2.F2.HA (clone-4 were plated at 8×10^4 cells per well in a six well plate. After 48hrs the cells were switched to serum-free media for 24hrs and subsequently stimulated with indicated amounts of AP1510 or 10 ng/ml EGF for 18-20hrs. Cells were trypsinized and resuspended in 1.0 ml of 10% serum containing medium and transferred to a tube

containing 10.0 ml of 1x PBS. The cells were pelleted by centrifugation at 1,200 rpm for 4 min. Pellets were washed again with 10.0 ml of 1x PBS and the resuspended in 0.5mls of 1x PBS. The resuspended cells were fixed overnight in 5.0 ml of ice-cold 100% ethanol. Ethanol was added very slowly, while vortexing, to avoid cell clumping. The fixed cells were pelleted and washed twice in 5.0 ml of 1x PBS containing 2.0% FBS. After the second wash the cells were resuspended in 0.5 mls of 1x PBS containing 2% FBS; 0.1% Tween-20; 20 µg/ml RNase A; and 10 µg/ml Propidium Iodide, incubated at 37°C for 2-3 hrs and analyzed by FACS. The data were analyzed by the ModFit program (Becton and Dickson) to calculate the percentage of cells in G0-G1, S and G2-M stages of cell cycle. The fold increase in the percentage of cells that are in S and G2-M phase was plotted.

Focus forming assay.

Rat1 fibroblasts were plated at 2×10^4 cells per well in a twelve well plate. The cells were infected with retroviruses expressing appropriate ErbB fusion at the rate of 150-250 CFU per well. Infected cells (24 hrs after infection) were trypsinized and replated on 10 cm plates containing different concentrations of AP1510. Cells from one infected well were plated onto a 10 cm plates in media containing 500 µg/ml G418 for 10-12 days. The focus assay was carried out 14 days by changing the drug-containing media once every three days. The plates were fixed with 4% formalin and stained with 4% Giemsa. Previous experiments have shown that AP1510 is active at least for 4 days as dilute aqueous solutions at 37°C (data not shown). For the experiment shown in Figure 7, Rat 1 cells were plated at 3×10^5 cells per 10 cm plate and infected with higher amount of virus stock and the exact number of CFU per plate was estimated by trypsinizing and plating one infected plate under 750 µg/ml G418.

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Results

Synthetic ligand-mediated ErbB dimerization.

To study the signaling and biological specificities of different ErbB dimers we have designed a dimerization strategy that employs synthetic bivalent dimerizing ligands (also called as Chemical Inducers of Dimerization, CIDs) and their binding proteins (Fig. 1, for a review see (67)). For homodimerization, we employed ligands that bind to the FK506 binding protein, FKBP12. The first reported homodimerizing compound, FK1012, was derived by chemical coupling of the monomeric FKBP ligand FK506 (70). Each molecule of FK1012 or a synthetic analog, AP1510 (2), binds to two copies of the ligand binding domain FKBP (Fig. 1A) and can induce homodimerization of proteins fused to FKBP. To induce heterodimerization, we used the natural product rapamycin that binds to one copy of FKBP and one copy of the FRB domain of FKBP-Rapamycin Associated Protein (FRAP) (Fig. 1A).

To create dimerizable ErbB receptors, the FKBP or FRB domains and an epitope tag were fused to the C-terminal end of ErbB cytoplasmic domain (Fig. 1C). To prevent binding of EGF family ligands released by autocrine secretion, the extracellular and transmembrane domains of the low affinity NGF receptor, p75, were substituted for the analogous domains of ErbB1 and B2. Using FKBP- or FRB-containing ErbB receptor chimeras it is possible to generate homodimers using AP1510 or heterodimers using rapamycin (Fig. 1D).

Dimerization of ErbB1 cytoplasmic domain using synthetic ligands result in a dose- dependent stimulation of receptor and substrate phosphorylation.

To establish whether synthetic ligands can be used to dimerize ErbB receptors we examined dimerization and activation of ErbB1 cytoplasmic domain (B1) fused to one copy of FKBP (F1) (denoted as p75.B1.F1.HA) (Fig. 1C). Stable cell lines expressing the p75.B1.F1.HA chimera were derived using Rat1 fibroblasts. AP1510, but not FK506, treatment resulted in a dose-dependent increase in tyrosine phosphorylation of cellular proteins

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(Fig. 2A compare lanes 1-6 and 7-8). As expected, the cells were still sensitive to EGF stimulation (Fig. 2A lane 9). Interestingly, the pattern of tyrosine phosphorylation after AP1510 stimulation was comparable to the pattern obtained after EGF stimulation (Fig. 2, compare lanes 6 and 9). For example, the adapter protein Shc and proteins with approximate molecular mass of 35, 42, 44, 60, 79 and 100 kDa (marked with asterisks) were phosphorylated by both EGF and AP1510 stimulation (Fig.2A). As expected, EGF stimulation resulted in phosphorylation of endogenous EGFR while AP1510 stimulation did not result in phosphorylation of any protein in the mobility range of EGFR. (Fig. 1A, compare lanes 2-6 and 9). Similar results were obtained in three independent Rat1 clones expressing p75.B1.F1.HA (data not shown). AP1510 stimulation of the parental Rat1 fibroblasts did not have any effect on tyrosine phosphorylation of cellular proteins (data not shown). To specifically examine the tyrosine phosphorylation status of the chimeric receptor, anti-HA immunoprecipitates were immunoblotted with anti-pTyr antibodies. The p75.B1.F1 chimera was inducibly tyrosine phosphorylated with maximal stimulation at 500 nM AP1510 (Fig. 2B lanes 1-6). As expected, neither the monomeric ligand nor EGF induced tyrosine phosphorylation of the ErbB1 chimera (Fig. 2B lanes 7-9). Synthetic ligand-mediated activation of the p75.B1.F1.HA receptor did not show any increase in tyrosine phosphorylation levels of the endogenous ErbB2 receptor (data not shown). Thus, activation of chimeric receptor appears to be independent of both EGF ligands and endogenous ErbB receptors.

Synthetic ligand-activated receptors are competent in recruiting signaling molecules and activating a downstream target.

Stimulation of wild type ErbB1 receptor by EGF results in recruitment of multiple cytoplasmic signaling molecules including Grb2 and Shc (1). To examine whether AP1510-activated chimeric ErbB1 receptors can recruit SH2-containing proteins, we immunoprecipitated the chimeric receptors and immunoblotted with antibodies to Grb2 and Shc

(Fig. 3A). Both Grb2 and Shc coimmunoprecipitated with tyrosine phosphorylated p75.B1.F1.HA in AP510-treated cells (Fig. 3A lanes 4-7) suggesting that synthetic ligand-activated ErbB1 receptors were competent to recruit known cytoplasmic signaling molecules.

Activation of EGF receptor is known to activate a signal transduction pathway leading to activation of extracellular signal regulated kinase 2 (Erk2 or MAPK). To test whether activation of the chimeric ErbB1 receptor results in activation of Erk2, p75.B1.F1.HA expressing cells or the parental Rat1 cells were stimulated with AP1510 and total cell lysates were immunoblotted with anti-Erk2 antibodies. Interestingly, AP1510 stimulation induced a characteristic mobility shift of Erk2 suggesting that synthetic ligand mediated dimerization of p75.B1.F1.HA leads to activation of downstream signaling targets.

Synthetic ligands activate other members of the ErbB family and the activated receptors retain their kinase specificity.

In order to examine whether dimerization of cytoplasmic domains can activate other ErbB family members and whether synthetic ligand-activated ErbB kinases retain their kinase specificity, we derived Rat1-based stable cell lines expressing AP1510-inducible ErbB2 chimeras. We found that cell lines expressing ErbB2 chimeras with one copy of FKBP (p75.B2.F1.HA) had very high levels of basal tyrosine phosphorylation (data not shown); however, cells expressing p75.B2.F2.HA, which contains two copies of FKBP (F2), had low levels of basal tyrosine phosphorylation and showed AP1510-inducible phosphorylation. To make an objective comparison between ErbB1 and ErbB2 homodimers, we derived cell lines expressing two-FKBP variants of either ErbB1 or ErbB2 (p75.B1.F2.HA or p75.B2.F2.HA). As observed with cells expressing the single FKBP ErbB1 chimera (p75.B1.F1.HA), addition of synthetic ligand to cells expressing the double FKBP ErbB1 chimera (p75.B1.F2.HA) resulted in increased phosphorylation of the chimeric receptor and selected proteins (Fig. 4A, lanes 1-3). AP1510 stimulation also resulted in tyrosine phosphorylation of the ErbB2 chimera, p75.B2.F2.HA, and other protein substrates (Fig. 4A, lanes 5-7).

In order to examine whether downstream signaling pathways are activated by the ErbB2 chimera, we examined the activation of Erk2 and Akt, a serine/threonine protein kinase that is activated by ErbB receptors (10).. Homodimerization of both ErbB1 and ErbB2 resulted in the characteristic mobility shift of Erk2 (Fig.4C). To examine activation of Akt, total cell lysates were immunoblotted with anti-Akt antibodies that specifically recognize Akt phosphorylated at serine 473 (Fig.4B). Phosphorylation of both Thr 308 and Ser 473 on Akt is required for full activation (19). AP1510 stimulation induced Ser473 phosphorylation in cells expressing either ErbB1 or ErbB2 chimeras (Fig. 4B). The difference in signal strength between lanes 1-4 and 5-8 (Fig.4A) is likely due to difference in the levels of protein loaded (compare lanes 1-4 and 5-8 in Fig.4B lower panel). It should be noted that both the cell lines used in this experiment express comparable levels of the ErbB chimera as determined by FACS analysis (see Materials and Methods).

It is known that c-Cbl is tyrosine phosphorylated only by ErbB1 and not by other ErbB family members (41). We examined c-Cbl tyrosine phosphorylation status upon synthetic ligand-mediated dimerization of either ErbB1 or ErbB2. Homodimerization of ErbB1 resulted in c-Cbl tyrosine phosphorylation (Fig. 4D), whereas phosphorylation of c-Cbl was barely detectable following ErbB2 homodimerization. This differential phosphorylation of c-Cbl was also observed in Cos7 cells transiently transfected with either p75.B1.F2.HA or p75.B2.F2.HA chimeras (data not shown). These observations suggest that dimerization of ErbB receptors activated by synthetic ligands can retain their kinase-specific functions as monitored by their ability to phosphorylate c-Cbl.

ErbB1 but not ErbB2 chimeras are internalized after AP1510 stimulation.

EGF activation has been shown to induce endocytosis of activated ErbB1 receptors (65). It has also been shown that ErbB chimeras containing the extracellular domain of ErbB1 and the cytoplasmic domains of ErbB2, ErbB3 or ErbB4 do not undergo EGF-induced receptor endocytosis (3). In order to determine whether synthetic ligand-activated ErbB

receptors undergo internalization, we stimulated p75.B1.F2.HA or p75.B2.F2.HA expressing

cells with AP1510 for different lengths of time and the cell surface proteins were subsequently

labeled with biotin at 4°C. The cell lysates were then subject to precipitation using

NeutrAvidin-coupled beads and the bound proteins were immunoblotted with HA tag

antibodies to determine the amount of chimeric receptor present at the cell surface. AP1510

treatment of ErbB1, (Fig. 5A lanes 5-7) but not ErbB2 (Fig. 5A lanes 12-14), expressing cells

resulted in a significant decrease in the amount chimeric receptors present at the cell surface.

This observation suggests that AP1510-stimulation results in internalization of activated ErbB1

receptors. The significant decrease in the levels of ErbB1 receptors at the cell surface after10

minutes of AP1510 stimulation is consistent with the internalization rates observed for the

peptide ligand, EGF (65). Stimulation of p75.B1.F2.HA expressing cells with the carrier alone

did not result in any change in the levels of ErbB1 chimera (Fig. 5A, lanes 1-4). Since

NeutrAvidin would precipitate all biotin labeled cell surface proteins the same blot was

reprobed with anti-beta1 integrin antibodies (Figure 5B) to demonstrate that the decrease in the

p75.B1.F2 levels was receptor-specific.

Immunofluorescent labeling of ErbB1 expressing cells with anti-p75 antibodies also

showed a ligand activation dependent internalization of the chimeric p75.B1.F2.HA receptor

(data not shown). These observations suggest that ErbB1 chimeras undergo synthetic ligand-

dependent endocytosis and also demonstrate that the ErbB chimeras retain their differential

regulation of receptor internalization.

Activation of ErbB homodimers results in induction of cell cycle progression.

To establish whether activation of ErbB receptors by synthetic ligands can induce cell

cycle progression, cells expressing either ErbB1 fused to one copy of FKBP (P75.B1.F1.HA)

or ErbB1 fused to two copies of FKBP (p75.B1.F2.HA) or ErbB2 fused to two copies of

FKBP (p75.B2.F2.HA) were starved in serum-free media for 24 hours. The cells were then

stimulated with indicated amounts of AP1510 for 16-18 hours and the DNA content was

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measured by Florescence Activated Cell Sorting (FACS) of propidium iodide-labeled cells

(Fig. 6). The fold increase in the percentage of cells that have left the G0-G1 stage of cell cycle

was calculated (see Materials and Methods). AP1510 stimulation of p75.B1.F1.HA,

p75.B1.F2.HA and p75.B2.F2.HA expressing cells resulted in a 1.5-2.0 fold increase and

EGF-stimulation resulted in a 1.8-2.5 fold increase in the percentage of cells that have left the

G0-G1 stage of cell cycle (Fig.6) These results suggest that ErbB1 and ErbB2 chimeras

activated using synthetic ligands were able to promote cell cycle progression.

ErbB1 and ErbB2 homodimers differ in their ability to induce focus formation in rat fibroblasts.

Since ErbB family members are known to be potent oncogenes, we examined

whether ErbB1 or ErbB2 homodimers can induce focus formation in fibroblasts. Rat1

fibroblasts were infected with retroviruses expressing either ErbB1 or ErbB2 fused to one or

two copies of FKBP (p75.B1.F1.HA, p75.B1.F2.HA, p75.B2.F1.HA or p75.B2.F2.HA).

The infected cells were maintained in the presence of different doses of AP1510 for 14 days.

The number of infected cells was determined by G418 selection since the virus carried the gene

encoding for neomycin (see Materials and Methods). Fifty five to sixty five percent of cells

infected with p75.B2.F2.HA retrovirus formed foci in the presence of AP1510 (Fig. 7, Table

1). In contrast, expression of ErbB1 chimera fused to two FKBP (p75.B1.F2.HA) showed

weak focus forming activity (Fig.7) with only 10% of the infected cells forming foci (Table 1).

In addition, the foci induced by ErbB1 chimera had a diffuse morphology and showed faint

Giemsa staining relative to the dense, intensely-stained foci induced by the ErbB2 chimera

(Fig. 7). Expression of ErbB2 chimera with one copy of FKBP (p75.B2.F1.HA) resulted in a

low level of ligand-independent focus formation, nevertheless showed a 6-7 fold ligand

inducibility (Fig. 7, Table 1). Surprisingly, ErbB1 chimera with one copy of FKBP

(p75.B1.F1.HA) failed to induce any detectable focus formation (Fig. 7, Table 1). The

difference in the focus inducing ability was not due to differences in expression levels of the

chimeric proteins since we detected comparable levels of expression by both anti-HA immunoblots and FACS analyses of the infected Rat1 cells stained with anti-p75 antibodies (data not shown).

Both ErbB1 and ErbB2 homodimers can induce a reversible morphological transformation of fibroblasts.

Transformed fibroblasts are known to display a refractile morphology and lose contact inhibition. We examined whether dimerization of the cytoplasmic domains of ErbB1 or ErbB2 induce morphological changes and whether these changes are reversible after ligand withdrawal. Stable cell lines expressing comparable levels of ErbB1 with one copy of FKBP (p75.B1.F1.HA) or ErbB1 with two copies of FKBP (p75.B1.F2.HA) or ErbB2 with two copies of FKBP (p75.B2.F2.HA) (see Materials and Methods) were grown in the presence of AP1510 for 48 hrs and the changes in morphology were recorded (Fig.8). Cells expressing p75.B1.F2.HA (g-i) or p75.B2.F2.HA (m-o), but not cells expressing p75.F1.HA (a p75 chimera without ErbB cytoplasmic domain, Fig. 1B) (a-c), lost their contact-inhibited flat morphology and assumed a transformed, refractile morphology in the presence of AP1510. Consistent with lack of focus forming ability, AP1510 did not induce morphological changes in cells expressing p75.B1.F1.HA (d-f).

After trypsinization and replating of the AP1510-treated cells in media without the ligand, the cells reverted to a non-transformed state, displaying a well-spread morphology and contact inhibition (j and p). This observation suggests that the morphological changes require continuous presence of the dimerizing ligand.

Synthetic ligand induced heterodimerization between ErbB receptors.

It has not been possible to study the signaling specificities of different ErbB heterodimers in isolation since most cell types express more than one ErbB family member. In order to establish whether synthetic ligands can be used to form heterodimers of selected ErbB

receptors, we generated a chimera containing the kinase-dead variant of ErbB1 (kdB1) fused to the FRB domain and a flag epitope tag (p75.kdB1.R1.Flag, see Materials and Methods). The p75.kdB1.R1.Flag chimera was not phosphorylated on tyrosine when expressed transiently in Cos7 cells (data not shown). Stable pools of Rat1 cells coexpressing the kinase-dead ErbB1-FRB chimera (p75.kdB1.R1.Flag) and either the wild type ErbB1-FKBP chimera (p75.B1.F2.HA) or the wild type ErbB2-FKBP chimera (p75.B2.F2.HA) were generated (Fig. 9A). As illustrated in Fig. 1D, addition of the FKBP-binding ligand, AP1510, to these cells should result in formation of either B1/B1 or B2/B2 homodimers while treatment with the heterodimerizing ligand, rapamycin, should result in either B1/kdB1 or B2/kdB1 heterodimers. As expected, AP1510 induced homodimerization and tyrosine phosphorylation of both ErbB1-FKBP (p75.B1.F2.HA, Fig. 9B, lanes 1-3) and ErbB2-FKBP (p75.B2.F2.HA, Fig 9B, lanes 7-9) chimeras. Interestingly, the kinase-dead ErbB1 chimera fused to the FRB domain, expressed in the same cell, was not tyrosine phosphorylated by AP1510 stimulation (Fig. 9C, lanes 1-3 and 7-9) likely due to the inability of AP1510 to bind FRB fused chimeras. However, rapamycin stimulation resulted in tyrosine phosphorylation of the kinase-dead ErbB1-FRB chimera (p75.kdB1.R1.Flag) (Fig. 9C, lanes 4-6, and 10-12) by both kinase-active ErbB1-FKBP (lanes 4-6) and kinase-active ErbB2-FKBP (lanes 10-12). Since rapamycin is known to dimerize a FKBP and a FRB domain (Fig. 1) this observation suggests that rapamycin can induce heterodimers between the kinase-dead ErbB1-FRB and kinase-active ErbB-FKBP receptors. Rapamycin stimulation did not change the phosphorylation status of either kinase-active ErbB1-FKBP (Fig. 9B, lanes 4-6) or kinase-active ErbB2-FKBP (Fig. 9B, lanes 10-12) possibly because the dimer comprises of one kinase active and one kinase dead receptor. This is consistent with the notion that that the tyrosine phosphorylation of ErbB receptors occur primarily by trans-phosphorylation within a dimer (76). The kinase active receptors observed in the anti-flag immunoprecipitates from rapamycin stimulated cell lysates (Fig.9C, lanes 4-6 and lanes 10-12) was due to the ability of rapamycin to induce a stable heterodimeric complex between the flag-tagged kinase dead and the weakly-phosphorylated

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kinase active ErbB receptors (Fig. 9B lanes 1 and 7). Since the tyrosine phosphorylation levels neither ErbB1 (Fig. 9B, compare lane 1 and lanes 4-6) nor ErbB2 (Fig. 9B, compare lane 7 and lanes 10-12) change in response to rapamycin stimulation it is unlikely that rapamycin stimulation affects the tyrosine phosphorylation status of FKBP-fused kinase-active ErbB chimeras. These results demonstrate that rapamycin can be used to form heterodimers in the absence of homodimers and AP1510 can be used to form homodimers in the absence of heterodimers.

c-Cbl can differentiate ErbB1 in homodimers from ErbB1 in ErbB1/ErbB2 heterodimers.

It is possible that heterodimers have different signaling specificity compared to homodimers. We tested whether the kinase-dead ErbB1 receptor phosphorylated by either a kinase-active ErbB1 (homodimer) or a kinase-active ErbB2 (heterodimer) differed in its ability to associate with cytoplasmic signaling molecules. Since c-Cbl has been shown to bind selectively to ErbB1 but not to other ErbB receptors (41), we asked whether c-Cbl can differentiate between the kinase-dead ErbB1 phosphorylated by a kinase-active ErbB1 receptor (homodimer) from a kinase-dead ErbB1 phosphorylated by a kinase-active ErbB2 receptor (heterodimer). The kinase-dead ErbB1 receptor fused to FRB domain (p75.kdB1.R1.Flag) was cotransfected with either kinase-active ErbB1-FKBP chimera (p75.B1.F2.HA) or kinase-active ErbB2-FKBP chimera (p75.B2.F2.HA) in Cos7 cells. In the presence of rapamycin the kinase active FKBP-fused ErbB1 and ErbB2 receptors coimmunoprecipitate with the kinase-dead ErbB1-FRB.Flag chimera (Fig. 9 and 10). Rapamycin stimulation resulted in increased phosphorylation of the kinase-dead ErbB1-FRB chimera (p75.kdB1.R1.Flag) by both ErbB1-FKBP (p75.B1.F2.HA) (Fig.10 lanes 1-3) and ErbB2-FKBP (p75.B2.F2.HA) (Fig.10, lanes 4-6) receptors as determined by anti-flag immunoprecipitation and anti-pTyr immunoblotting. Endogenous c-Cbl was also immunoprecipitated from the same cell lysates and the precipitates were blotted with anti-pTyr antibodies (Fig.10 lanes 7-12) to identify the ErbB chimeras that

bind to c-Cbl. c-Cbl was able to coimmunoprecipitate the tyrosine phosphorylated, kinase-dead, ErbB1-FRB chimera (p75.kdB1.R1.Flag) only when the kinase-dead ErbB1 was phosphorylated by kinase-active ErbB1 chimera (p75.B1.F2.HA) (Fig.10 lanes 7-9) but not when kinase-dead ErbB1 was phosphorylated by kinase-active ErbB2 chimera (p75.B2.F2.HA) (Fig.10 lanes 10-12). This observations suggest that c-Cbl can differentiate ErbB1 molecules in a homodimer (p75.B1.F2/p75.kdB1.R1) from ErbB1 molecules in a heterodimer with ErbB2 (p75.B2.F2/p75.kdB1.R1).

Discussion

We demonstrate that synthetic dimerizing ligands can selectively activate homo and heterodimers of the ErbB family of receptors and result in activation of signal transduction pathways. Synthetic ligand-mediated dimerization and activation also induces a dose-dependent stimulation of phenotypic alterations known to be regulated by ErbB receptors (e.g. stimulation of cell proliferation, morphological transformation and foci formation). In addition, using a heterodimerizing ligand we demonstrate that c-Cbl can associate with ErbB1 in an ErbB1/ErbB1 homodimer but does not associate with ErbB1 in an ErbB1/ErbB2 heterodimer. These observations suggest that this approach can be used to study the signaling and biological specificities of ErbB homodimers and heterodimers and provide the first clear evidence for differential signaling by ErbB homo and heterodimers.

The extracellular cysteine-rich domains of ErbB receptors play important roles in ligand binding and receptor dimerization (11, 12, 14, 59, 60, 66). Mutation or addition of Cys residues at the juxtamembrane region and generation of unpaired Cys residues results in dimerization of ErbB2 (12, 60). Interestingly, a number of such dimers fail to induce transformation suggesting that forced dimerization is not sufficient for functional activation of ErbB2 (11). The transmembrane and the juxtamembrane regions of ErbB2 form a helical structure and receptor dimerization is thought to promote a helix-helix interaction (12). It is proposed that some of the Cys modification-induced dimerization may result in packing the

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helices in an "unfavorable or non-permissive" orientation for signaling (12). Results presented in this report suggest that dimerization of the cytoplasmic domain was sufficient to activate both ErbB1 and ErbB2 receptors. This is consistent with an earlier report which suggests that membrane localization of the cytoplasmic domain of ErbB2 was sufficient to induce kinase activation and transformation (26). It will be interesting to progressively change the orientation/location of the synthetic ligand binding domains within the chimera to ask whether the ErbB cytoplasmic domains requires a specific dimerization interface.

Synthetic ligand-inducible dimerization was able to activate both biochemical and biological processes that are known to be stimulated by ErbB receptors. Both ErbB1 and ErbB2 homodimers were able to induce activation of the serine threonine kinase Akt (Fig. 4). Akt is a known downstream target of activated PI 3'-kinase. It is unclear how either of these homodimers activate the PI 3'-kinase pathway since neither ErbB1 nor ErbB2 possess the binding site for the SH2 domain of p85 subunit of PI 3'-kinase (24, 39, 53, 64). It is possible that c-Cbl mediates the ErbB1 homodimer-induced activation of PI 3'-kinase (63), whereas, c-Cbl is unlikely to play a role in ErbB2 homodimer-induced activation of the PI 3'-kinase pathway since ErbB2 does not show strong association with c-Cbl (Fig. 4 and 8). Further experiments will be necessary to understand the underlying mechanism leading to activation of PI 3'-kinase by ErbB1 and ErbB2 homodimers.

Activation of ErbB1 or ErbB2 dimers using synthetic ligands resulted in a ligand-dependent acquisition of transformed-cell morphology while removal of AP1510 caused a reversion to a normal morphology. These observations demonstrate the continuous requirement of dimerization signal for maintenance of the transformed morphology. It will be of interest to determine whether the ligand–dependent transformation and reversion can be induced in animal models where other mutations are involved during tumorigenesis. Development of such a model with inducible activation of specific homo and heterodimers of different ErbB receptors would be very useful in understanding the early events of tumor progression in adult animals.

The biological effects of ErbB1 homodimerization in fibroblasts or other cell types is unclear since almost all cell types express more than one member of the ErbB receptor family. Previous reports have shown that ErbB1 is able to induce foci formation in a ligand-dependent manner in NIH 3T3 clones which either lack ErbB1 (21) or lack expression of all ErbB family members (79) and that EGF-activated ErbB1 possesses weaker foci forming activity than activated ErbB2 (21). It is possible that these cell lines are not devoid of ErbB receptors and the observed phenotype may be a result heterodimers involving ErbB1. Studies using ErbB receptor-deficient hemopoietic cells that require IL-3 for survival and growth have suggested that ErbB1 homodimers are not effective in inducing proliferation but can induce IL-3-independent survival (56). The approach presented here enables us to study the effect of homodimers in the absence of lateral/combinatorial interactions with other ErbB family members in cells that naturally express these receptors; hence, we believe that our observations provide a clear demonstration of the biological differences between ErbB1 and ErbB2 homodimers in fibroblasts (see below).

It is unclear why synthetic ligand-induced ErbB1 homodimers possess 5-7 fold weaker foci forming activity compared to ErbB2 homodimers. It is possible that ErbB1 homodimers do not activate signaling molecules that mediate transformation as effectively as ErbB2 (58). Alternatively, the homodimers may couple to negative regulators of signaling. Heterodimerization of ErbB1 with other ErbB receptors can either generate novel autophosphorylation sites for activating cytoplasmic signaling molecules or may fail to generate certain autophosphorylation sites to preclude interactions with negative regulator(s). We present evidence which suggests that ErbB1 homodimers and ErbB1/ErbB2 heterodimers differ in their ability to recruit a cytoplasmic signaling protein, c-Cbl. It is possible that such differences may play role in determining the biological specificity of homo and heterodimers.

c-Cbl has been implicated both as a positive and negative regulator of cell signaling (44, 47). The mechanism by which Cbl functions is not known. Recent observations suggest that c-Cbl promotes ubiquitination and degradation of activated EGF and PDGF receptors (42, 47).

Interestingly, only ErbB1, and not ErbB3, is ubiquitinated and downregulated by c-Cbl and this is dependent on the presence of ErbB1 cytoplasmic tail (42). It is possible that ErbB1 homodimers and ErbB1/ErbB2 heterodimers are differentially ubiquitinated and downregulated. Such a possibility is consistent with the observation that ErbB1 homodimers are endocytosed (Figure 5) and degraded while ErbB1/ErbB2 heterodimers are recycled to the membrane after EGF stimulation (40). However, the differential association with c-Cbl may also regulate multiple downstream signaling pathways that play a role in signaling by ErbB1, ErbB2 homo and heterodimers.

It is unclear why ErbB1 chimeras with one copy of FKBP (p75.B1.F1) did not induce morphological changes or focus formation (Fig. 7 and 8). One copy of FKBP was sufficient to activate the receptor since AP1510 induced tyrosine phosphorylation of multiple cellular proteins including Erk2 (Fig. 2) as well as stimulation of DNA synthesis in cells expressing p75.B1.F1.HA chimera (Fig.6). In addition, AP1510-activation of the single FKBP version of the ErbB2 chimera, p75.B2.F1, results in induction of focus formation (see Fig. 7 and Table 1). It is possible that simple dimerization of ErbB1 is not sufficient for morphological transformation whereas dimerization of ErbB2 is sufficient. Consistent with that possibility, the Val664-Glu mutation in ErbB2 promotes homodimerization, activation of the kinase and transformation of fibroblasts (75). Interestingly, insertion of a similar mutation into ErbB1 does not result in ligand-independent transformation of fibroblasts (15, 38, 46) suggesting that homodimerization of ErbB1 may not be sufficient for transformation. Our results suggest that p75.B1.F2.HA chimera which contains two copies of FKBP can induce only a weak transformation. It should be noted that two FKBP containing chimeras can form higher-order complexes however, sucrose gradient centrifugation of p75.B1.F2.HA expressing cell lysate showed a ligand-dependent formation of a dimeric complex (data not shown). Further experiments are required to better understand the difference between the ErbB1 chimeras consisting of one or two copies of FKBP.

We will not be able to use the heterodimerizing ligand, rapamycin, in biological studies since rapamycin is a known immunosuppressive drug that can negatively regulate cellular kinases FRAP and p70^{S6K}. However, synthetic versions of rapamycin (rapalogs) have been generated which do not bind endogenous FRAP and instead can only bind to a FRAP molecule that has been appropriately engineered to fit the modification on rapamycin (18a, 43). These 'rapalogs' possess no immunosuppressive functions (18a, 43). We are in the process of constructing ErbB chimeras with the modified FRB domain which will enable us to study the biological effects of distinct ErbB heterodimers.

To our knowledge the results presented here provide the first direct evidence for differential signaling by ErbB1 homodimers and ErbB1/ErbB2 heterodimers. It will be of interest to apply this strategy to understand the signaling specificities of different ErbB receptor homo- and heterodimers. Since synthetic ligand mediated activation of chimeric ErbB receptors is independent of the endogenous levels of ErbB receptor expression this approach could be well suited to study the biological effects of different ErbB receptor dimers in the cell type of choice.

Acknowledgments.

Figure Legends

Figure 1. Synthetic ligands for ErbB dimerization.

The ligand binding domains FKBP12 and FKBP-Rapamycin Binding domain (FRB) (A) were subcloned as single or double copies to generate expression vectors in panel B. The intracellular domain of ErbB receptors were PCR amplified and subcloned into the expression vectors (B) as described under Materials and Methods. Addition of AP1510 to cells expressing a FKBP fused ErbB receptors will result in generation of 'homodimers' (D), while addition of rapamycin to cells coexpressing ErbB1-FKBP chimera (p75.B1.F1.HA) and ErbB2-FRB chimera (p75.B2.R1.Flag) will result in a p75.B1.F1.HA/p75.B2.R1.Flag heterodimer (D).

Figure 2. Dimerization of ErbB1 cytoplasmic domain using synthetic ligands results in a dose-dependent stimulation of receptor and substrate phosphorylation.

Rat1 fibroblasts expressing ErbB1 fused to one copy of FKBP (p75.B1.F1.HA) were stimulated with increasing amounts of AP1510 (nM) (lanes 2-6) or FK506 (lanes 7-8) for 15 minutes or stimulated with 50ng/ml of EGF for 5 minutes. Cell lysates were collected and 45 μg of protein was resolved and immunoblotted with anti-phosphotyrosine (anti-pTyr) antibodies (A). The blot was stripped to reprobed with anti-Shc antibodies and the p46 and p52 isoforms of Shc and other cellular proteins that were tyrosine phosphorylated by ligand stimulation are indicated by asterisks. 700 μg of lysate was used for immunoprecipitation with anti-HA antibodies and immunoblotted with anti-pTyr (B, upper panel). The anti-pTyr blot was subsequently stripped and reprobed with anti-EGFR antibodies (B, lower panel).

Figure 3. Synthetic ligand activated receptors are competent in recruiting signaling molecules and activating a downstream target.

HA epitope-containing proteins were immunoprecipated (lanes 4-7) from 500 µg of cell lysate and the membrane was probed with antibodies against anti-pTyr (A. first panel), anti-Grb2 (A, second panel), or anti-Shc (A, third panel). The blot from the first panel was stripped and reprobed with anti-EGFR (A, fourth panel). The normal mouse serum (NMS, lanes 1-3) was used as a nonspecific control. Total cell lysate from p75.B1.F1.HA or Rat1 cells stimulated with AP1510 (nM) were immunoblotted with anti-Erk2 antibodies (B).

Figure 4. Synthetic ligands can activate other members of the ErbB family and the activated receptors retain their kinase specificity.

Total cell lysates from cells stimulated with AP1510 (nM) or EGF (50 ng/ml) were resolved and blotted with anti-pTyr antibodies (A). The positions of p75.B1.F2.HA, p75.B2.F2.HA and Shc are indicated. Top two third of the blot in panel A was stripped and reprobed with anti-phospho-473 Akt antibody (B, upper panel) and the blot was re-stripped and blotted with anti-Akt antibody (B, lower panel). The lower third of the blot in panel A was stripped and reprobed with anti-Erk2 (C). c-Cbl was immunoprecipitated from 750 µg of lysate and immunoblotted with anti-pTyr antibodies (D, upper panel) and the blot was subsequently stripped and reprobed with anti-Cbl antibodies (D, lower panel). The position of the coimmunoprecipitated p75.B1.F2.HA is indicated.

Figure 5. ErbB1 but not ErbB2 chimeras are internalized after AP1510 stimulation.

Cell lines expressing either ErbB1 or ErbB2 chimeras were stimulated with carrier alone (ethanol) or with 500nM of AP1510 for indicated lengths of time. The cell surface proteins were subsequently biotinylated by incubating with NHS-S-S-Biotin at 4°C for 1hr. The

biotinylated proteins were immunoprecipitated using sepharose conjugated NeutrAvidin beads and immunoblotted with either anti-HA (A) or with anti-beta1 integrin (B) antibodies.

Figure 6. Activation of ErbB homodimers results in induction of cell cycle progression.

The parental Rat1 cells or cell lines expressing different ErbB chimeras were serum starved, stimulated and analyzed by FACS as described under Materials and Methods. The graph represents fold increase in the percentage of cells that have left G0-G1 stage of cell cycle. The parental Rat1 cells (open squares), cells expressing ErbB1 with one copy FKBP (p75.B1.F1) (closed squares), cells expressing ErbB1 with two copies of FKBP (p75.B1.F2) (open circles) and cells expressing ErbB2 with two copies of FKBP (p75.B2.F2) (open triangles) were used for the analysis. EGF was used at 10 ng/ml concentration.

Figure 7. ErbB1 and ErbB2 homodimers differ in their ability to induce foci formation in Rat fibroblasts.

Rat1 cells were infected with retroviruses containing different ErbB fusion. The cells were maintained in media containing indicated amounts of AP1510 (nM) for 14 days and fixed and stained with Giemsa stain. One set of infected cells were trypsinized and 1/10th of the cells were replated in media containing G418 to ascertain the number of CFU infected per plate.

Figure 8. Both ErbB1 and ErbB2 homodimers are able to induce reversible morphological transformation of fibroblasts.

The cells were plated in the presence of AP1510 (nM) and allowed to grow for 48hrs. The morphology of the cells were recorded (a-i and m-o). The cells in 'i' and 'o' were trypsinized and replated either in media without (j and p) or with AP1510 (k,l,q and r). P75.F1 (Fig. 1B) corresponds to cells expressing the chimera without ErbB cytoplasmic domain.

Figure 9. Synthetic ligand induced heterodimerization between ErbB receptors.

P75.B1.F2.HA and p75.B2.F2.HA expressing cells were transfected with p75.kdB1.R1.Flag and stable pools containing B1.F2.HA + kdB1.R1.Flag and B2.F2.HA + kdB1.R1.Flag were selected. The relative expression levels of each chimera in both the pools were examined by immunoblotting cell lysates with either anti-HA or anti-Flag antibodies (A). The parental Rat1 cell lysate was used as negative control. The pools were either stimulated with AP1510 (nM) (lanes 2-3 and 8-9) or with rapamycin (nM) (lanes 4-6 and 10-12) and immunoprecipitated with either anti-HA (B) or anti-Flag (C) antibodies and immunoblotted with anti-pTyr antibodies. The blots in the upper panel of B and C were stripped and reprobed with anti-HA or anti-Flag antibodies respectively (B and C, lower panels).

Figure 10. c-Cbl can differentiate ErbB1 in homodimers from ErbB1 in ErbB1/ErbB2 heterodimers.

Cos7 cells were cotransfected with p75.kdB1.R1.Flag and p75.B1.F2.HA (lanes 1-3 and 7-9) or p75.B2.F2.HA (lanes 4-6 and 10-12). The cells were stimulated with indicated amounts of rapamycin (nM) and 1.5 mg of lysates was used for immunoprecipitation with anti-Flag antibodies (lanes 1-6) or with anti-Cbl (lanes 7-12) antibodies and immunoblotted with anti-pTyr antibodies (upper panel). The c-Cbl portion of the blot (lanes 7-12) was stripped and reprobed with anti-Cbl antibodies (lower panel)

TABLE.1: Transformation of Rat1 fibroblasts by ErbB1 or ErbB2 homodimers*.

AP1510 ErbB chimera	0	50nM	100nM	250nM	500nM	1000nM
p75.B1.F1	0	0	0	0	0	1
p75.B1.F2	0	2 ± 1	7 ± 3	10 ± 4	8 ± 1	7 ± 1
p75.B2.F1	6 ± 1	6 ± 2	8 ± 1	19 ± 2	47 ± 6	34 ± 10
p75.B2.F2	1	16 ± 3	38 ± 7	55 ± 3	62 ± 9	58 ± 6

*Number of foci per 100 CFU.
Each value represents an average of at least three independent experiments

References.

1.   **Alroy, I., and Y. Yarden.** 1997. The ErbB signaling network in embyrogensis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. FEBS Lett **410:**83-86.

2.   **Amara, J. F., T. Clackson, V. M. Rivera, T. Guo, T. Keenan, S. Natesan, R. Pollock, W. Yang, N. L. Courage, D. A. Holt, and M. Gilman.** 1997. A versatile synthetic dimerizer for the regulation of protein-protein interactions. Proc Natl Acad Sci U S A **94:**10618-23.

3.   **Baulida, J., M. H. Kraus, M. Alimandi, P. P. Di Fiore, and G. Carpenter.** 1996. All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. J Biol Chem **271:**5251-7.

4.   **Beerli, R. R., D. Graus-Porta, K. Woods-Cook, X. Chen, Y. Yarden, and N. E. Hynes.** 1995. Neu differentiation factor activation of ErbB3 and ErbB4 is cell specific and displays a differential requirement for ErbB2. Mol. Cell. Biol. **15:**6496-6705.

5.   **Belsches, A. P., M. D. Haskell, and S. J. Parsons.** 1997. Role of c-Src tyrosine kinase in EGF-induced mitogenesis. Front Biosci **2:**d501-18.

6.   **Birge, R. B., B. S. Knudsen, D. Besser, and H. Hanafusa.** 1996. SH2 and SH3-containing adaptor proteins: redundant or independent mediators of intracellular signal transduction. Genes Cells **1:**595-613.

7.   **Blau, C. A., K. R. Peterson, J. G. Drachman, and D. M. Spencer.** 1997. A proliferation switch for genetically modified cells. Proc Natl Acad Sci U S A **94:**3076-81.

8.   **Boyer, B., S. Roche, M. Denoyelle, and J. P. Thiery.** 1997. Src and Ras are involved in separate pathways in epithelial cell scattering. Embo J **16:**5904-13.

9.   **Burden, S., and Y. Yarden.** 1997. Neuregulins and their receptors: A versatile signaling module in organogenesis and oncogenesis. Neuron **18:**847-855.

10.   **Burgering, B. M., and P. J. Coffer.** 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction [see comments]. Nature **376:**599-602.

11.   **Burke, C. L., M. A. Lemmon, B. A. Coren, D. M. Engelman, and D. F. Stern.** 1997. Dimerization of the p185neu transmembrane domain is necessary but not sufficient for transformation. Oncogene **14:**687-96.

12.   **Burke, C. L., and D. F. Stern.** 1998. Activation of Neu (ErbB-2) mediated by disulfide bond-induced dimerization reveals a receptor tyrosine kinase dimer interface. Mol Cell Biol **18:**5371-9.

13.   **Campbell, S. L., R. Khosravi-Far, K. L. Rossman, G. J. Clark, and C. J. Der.** 1998. Increasing complexity of Ras signaling. Oncogene **17:**1395-413.

14.   **Cao, H., L. Bangalore, C. Dompe, B. J. Bormann, and D. F. Stern.** 1992. An extra cysteine proximal to the transmembrane domain induces differential cross-linking of p185neu and p185neu. J Biol Chem **267:**20489-92.

15.   **Carpenter, C. D., H. A. Ingram, C. Cochet, G. M. Walton, C. S. Lazar, J. M. Sowadski, M. G. Rosenfeld, and G. N. Gill.** 1991. Structural analysis of the transmembrane domain of the epidermal growth factor receptor. J. Biol. Chem. **266:**5750-5755.

16.   **Carpenter, G.** 1992. Receptor tyrosine kinase substrates: src homology domains and signal transduction. Faseb J **6:**3283-9.

17.   **Carraway, K. L. r.** 1996. Involvement of the neuregulins and their receptors in cardiac and neural development. Bioessays **18:**263-6.

18.   **Carraway, K. L. 3rd., and L. C. Cantley.** 1994. A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. Cell **78:**5-8.

18a.   **Clackson, T.** Personal communication.

19.   **Coffer, P. J., J. Jin, and J. R. Woodgett.** 1998. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation [In Process Citation]. Biochem J **335:**1-13.

19a   **Crovello, C.S., K.L. Carraway 3rd.** Personal communication.

20.   **Daly, R. J.** 1998. The Grb7 family of signalling proteins [In Process Citation]. Cell Signal **10:**613-8.

21.   **Di Fiore, P. P., O. Segatto, W. G. Taylor, S. A. Aaronson, and J. H. Pierce.** 1990. EGF receptor and *erb*B-2 tyrosine kinase domains confer cell specificity for mitogenic signaling. Science **248:**79-83.

22.   **Fazioli, F., L. Minichiello, V. Matoska, P. Castagnino, T. Miki, W. T. Wong, and P. P. Di Fiore.** 1993a. Eps8, a substrate for the epidermal growth factor receptor kinase, enhances EGF-dependent mitogenic signals. Embo J **12:**3799-808.

23.   **Fazioli, F., L. Minichiello, B. Matoskova, W. T. Wong, and P. P. Di Fiore.** 1993. eps15, a novel tyrosine kinase substrate, exhibits transforming activity. Mol Cell Biol **13:**5814-28.

24.   **Fedi, P., J. H. Pierce, P. P. di Fiore, and M. H. Kraus.** 1994. Efficient coupling with phosphatidylinositol 3-kinase, but not phospholipase C gamma or GTPase-activating protein, distinguishes ErbB- 3 signaling from that of other ErbB/EGFR family members. Mol Cell Biol **14:**492-500.

25.   **Feng, G. S., and T. Pawson.** 1994. Phosphotyrosine phosphatases with SH2 domains: regulators of signal transduction. Trends Genet **10:**54-8.

26.   **Flanagan, J. G., and P. Leder.** 1988. neu protooncogene fused to an immunoglobulin heavy chain gene requires immunoglobulin light chain for cell surface expression and oncogenic transformation. Proc Natl Acad Sci U S A **85:**8057-61.

27.   **Fowler, K. J., F. Walker, W. Alexander, M. L. Hibbs, E. C. Nice, R. M. Bohmer, G. B. Mann, C. Thumwood, R. Maglitto, J. A. Danks, and et al.** 1995. A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation. Proc Natl Acad Sci U S A **92:**1465-9.

28.   **Gassmann, M., and G. Lemke.** 1997. Neuregulins and neuregulin receptors in neural development. Curr Opin Neurobiol **7:**87-92.

29. **Graus Porta, D., R. R. Beerli, and N. E. Hynes.** 1995. Single-chain antibody-mediated intracellular retention of ErbB-2 impairs Neu differentiation factor and epidermal growth factor signaling. Mol Cell Biol **15:**1182-91.

30. **Graus-Porta, D., R. R. Beerli, J. M. Daly, and N. E. Hynes.** 1997. ErbB-2 the preferred heterodimerization partener of all ErbB receptors, is a mediator of lateral signaling. EMBO J **16:**1647-1655.

31. **Hato, T., N. Pampori, and S. J. Shattil.** 1998. Complementary roles for receptor clustering and conformational change in the adhesive and signaling functions of integrin alphaIIb beta3. J Cell Biol **141:**1685-95.

32. **Hijazi, M. M., P. E. Young, M. K. Dougherty, D. S. Bressette, T. T. Cao, J. H. Pierce, L. M. Wong, M. Alimandi, and C. R. King.** 1998. NRG-3 in human breast cancers: activation of multiple erbB family proteins [In Process Citation]. Int J Oncol **13:**1061-7.

33. **Ho, S. N., S. R. Biggar, D. M. Spencer, S. L. Schreiber, and G. R. Crabtree.** 1996. Dimeric ligands define a role for transcriptional activation domains in reinitiation. Nature **382:**822-6.

34. **Holsinger, L. J., D. M. Spencer, D. J. Austin, S. L. Schreiber, and G. R. Crabtree.** 1995. Signal transduction in T lymphocytes using a conditional allele of Sos. Proc Natl Acad Sci U S A **92:**9810-4.

35. **Hoschuetzky, H., H. Aberle, and R. Kemler.** 1994. Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. J Cell Biol **127:**1375-80.

36. **Hynes, N., and D. F. Stern.** 1994. The biology of *erb-2/neu/HER-2* and its role in cancer. Biochem. Biophys. Acta **1198:**165-184.

37. **Karunagaran, D., E. Tzahar, R. R. Beerli, X. Chen, D. Graus-Porta, B. J. Ratzkin, R. Seger, N. E. Hynes, and Y. Yarden.** 1996. ErbB-2 is a common

auxiliary subunit of NDF and EGF receptors: implications for breast cancer. Embo J **15:**254-64.

38.   **Kashles, O., D. Szapary, F. Bellot, A. Ullrich, J. Schlessinger, and A. Schmidt.** 1988. Ligand-induced stimulation of epidermal growth factor receptor mutants with altered transmembrane regions. Proc Natl Acad Sci U S A **85:**9567-71.

39.   **Kim, H. H., S. L. Sierke, and J. G. Koland.** 1994. Epidermal growth factor-dependent association of phosphatidylinositol 3-kinase with the erbB3 gene product. J Biol Chem **269:**24747-55.

40.   **Lenferink, A. E., R. Pinkas-Kramarski, M. L. van de Poll, M. J. van Vugt, L. N. Klapper, E. Tzahar, H. Waterman, M. Sela, E. J. van Zoelen, and Y. Yarden.** 1998. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. Embo J **17:**3385-97.

41.   **Levkowitz, G., L. N. Klapper, E. Tzahar, A. Freywald, M. Sela, and Y. Yarden.** 1996. Coupling of the c-Cbl protooncogene product to ErbB-1/EGF-receptor but not to other ErbB proteins. Oncogene **12:**1117-25.

42.   **Levkowitz, G., H. Waterman, E. Zamir, Z. Kam, S. Oved, W. Y. Langdon, L. Beguinot, B. Geiger, and Y. Yarden.** 1998. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev **12:**3663-3674.

43.   **Liberles, S. D., S. T. Diver, D. J. Austin, and S. L. Schreiber.** 1997. Inducible gene expression and protein translocation using nontoxic ligands identified by a mammalian three-hybrid screen. Proc Natl Acad Sci U S A **94:**7825-30.

44.   **Liu, Y., and A. Altman.** 1998. Cbl: Complex formation and functional implications. Cell. Signal. **10:**377-385.

45.   **MacCorkle, R. A., K. W. Freeman, and D. M. Spencer.** 1998. Synthetic activation of caspases: artificial death switches. Proc Natl Acad Sci U S A **95:**3655-60.

46.   **Miloso, M., M. Mazzotti, W. C. Vass, and L. Beguinot.** 1995. SHC and GRB-2 are constitutively by an epidermal growth factor receptor with a point mutation in the transmembrane domain. J Biol Chem **270:**19557-62.

47.   **Miyake, S., M. L. Lupher, Jr., C. E. Andoniou, N. L. Lill, S. Ota, P. Douillard, N. Rao, and H. Band.** 1997. The Cbl protooncogene product: from an enigmatic oncogene to center stage of signal transduction. Crit Rev Oncog **8:**189-218.

48.   **Morgenstern, J. P., and H. Land.** 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res **18:**3587-96.

49.   **Muthuswamy, S. K., and W. J. Muller.** 1995. Direct and specific interaction of c-Src with Neu is involved in signaling by the epidermal growth factor receptor. Oncogene **11:**271-9.

50.   **Ojaniemi, M., and K. Vuori.** 1997. Epidermal growth factor modulates tyrosine phosphorylation of p130Cas. Involvement of phosphatidylinositol 3'-kinase and actin cytoskeleton. J Biol Chem **272:**25993-8.

51.   **Olayioye, M. A., D. Graus-Porta, R. R. Beerli, J. Rohrer, B. Gay, and N. E. Hynes.** 1998. ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. Mol Cell Biol **18:**5042-51.

52.   **Pinkas Kramarski, R., L. Soussan, H. Waterman, G. Levkowitz, I. Alroy, L. Klapper, S. Lavi, R. Seger, B. J. Ratzkin, M. Sela, and Y. Yarden.** 1996. Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. Embo J **15:**2452-67.

53.   **Prigent, S. A., and W. Gullick.** 1994. Identification of c-erbB-3 binding sites for phosphotidyl 3'-kinase and SHC using an EGF receptor/c-erbB-3 chimera. EMBO J. **13:**2831-2841.

54.   **Rankin, S., R. Hooshmand-Rad, L. Claesson-Welsh, and E. Rozengurt.** 1996. Requirement for phosphatidylinositol 3'-kinase activity in platelet- derived growth

factor-stimulated tyrosine phosphorylation of p125 focal adhesion kinase and paxillin. J Biol Chem **271:**7829-34.

55.   **Riese, D. J. I., and D. F. Stern.** 1998. Specificity within the EGF family/ErbB receptor family signaling network. Bioessays **20:**41-48.

56.   **Riese II, D. J., T. M. van Raaji, G. D. Plowman, G. C. Andrews, and D. Stern.** 1995. The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. Mol. Cell. Biol. **15:**5770-5776.

57.   **Rivera, V. M., T. Clackson, S. Natesan, R. Pollock, J. F. Amara, T. Keenan, S. R. Magari, T. Phillips, N. L. Courage, F. Cerasoli, Jr., D. A. Holt, and M. Gilman.** 1996. A humanized system for pharmacologic control of gene expression [see comments]. Nat Med **2:**1028-32.

58.   **Romano, A., W. T. Wong, M. Santoro, P. J. Wirth, S. S. Thorgeirsson, and P. P. Di Fiore.** 1994. The high transforming potency of erbB-2 and ret is associated with phosphorylation of paxillin and a 23 kDa protein. Oncogene **9:**2923-33.

59.   **Siegel, P. M., D. L. Dankort, W. R. Hardy, and W. J. Muller.** 1994. Novel activating mutations in the neu proto-oncogene involved in induction of mammary tumors. Mol Cell Biol **14:**7068-77.

60.   **Siegel, P. M., and W. J. Muller.** 1996. Mutations affecting conserved cysteine residues within the extracellular domain of Neu promote receptor dimerization and activation. Proc Natl Acad Sci U S A **93:**8878-83.

61.   **Skolnik, E. Y., B. Margolis, M. Mohammadi, E. Lowenstein, R. Fischer, A. Drepps, A. Ullrich, and J. Schlessinger.** 1991. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell **65:**83-90.
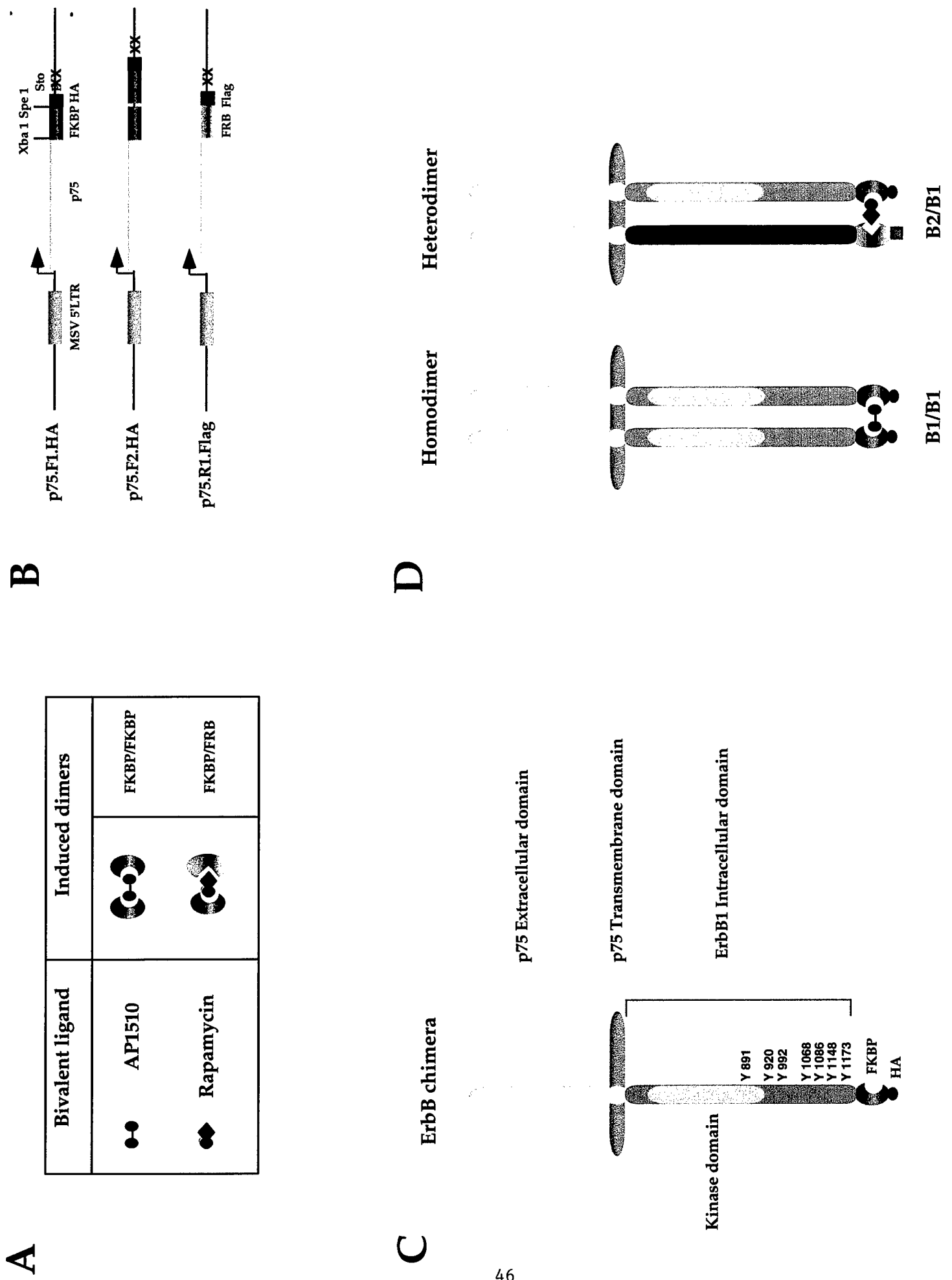
62.   **Sliwkowski, M. X., G. Schaefer, R. W. Akita, J. A. Lofgren, V. D. Fitzpatrick, A. Nuijens, B. M. Fendly, R. A. Cerione, R. L. Vandlen, and K.**

L. Carraway, 3rd. 1994. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J Biol Chem **269:**14661-5.

63.   **Soltoff, S. P., and L. C. Cantley.** 1996. p120cbl is a cytosolic adapter protein that associates with phosphoinositide 3-kinase in response to epidermal growth factor in PC12 and other cells. J Biol Chem **271:**563-7.

64.   **Soltoff, S. P., K. L. r. Carraway, S. A. Prigent, W. G. Gullick, and L. C. Cantley.** 1994. ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. Mol Cell Biol **14:**3550-8.

65.   **Sorkin, A., and C. M. Waters.** 1993. Endocytosis of growth factor receptors. Bioessays **15:**375-382.

66.   **Sorokin, A., M. A. Lemmon, A. Ullrich, and J. Schlessinger.** 1994. Stabilization of an active dimeric form of the epidermal growth factor receptor by introduction of an inter-receptor disulfide bond. J Biol Chem **269:**9752-9.

67.   **Spencer, D. M.** 1996. Creating conditional mutations in mammals. Trends Genet **12:**181-7.

68.   **Spencer, D. M., P. J. Belshaw, L. Chen, S. N. Ho, F. Randazzo, G. R. Crabtree, and S. L. Schreiber.** 1996. Functional analysis of Fas signaling in vivo using synthetic inducers of dimerization. Curr Biol **6:**839-47.

69.   **Spencer, D. M., I. Graef, D. J. Austin, S. L. Schreiber, and G. R. Crabtree.** 1995. A general strategy for producing conditional alleles of Src-like tyrosine kinases. Proc Natl Acad Sci U S A **92:**9805-9.

70.   **Spencer, D. M., T. J. Wandless, S. L. Schreiber, and G. R. Crabtree.** 1993. Controlling signal transduction with synthetic ligands [see comments]. Science **262:**1019-24.

71.   **Tzahar, E., R. Pinkas-Kramarski, J. D. Moyer, L. N. Klapper, I. Alroy, G. Levkowitz, M. Shelly, S. Henis, M. Eisenstein, B. J. Ratzkin, M.**

Sela, G. C. Andrews, and Y. Yarden. 1997. Bivalence of EGF-like ligands drives the ErbB signaling network. Embo J **16:**4938-50.

72.   **Tzahar, E., H. Waterman, X. Chen, G. Levkowitz, D. Karunagaran, S. Lavi, B. J. Ratzkin, and Y. Yarden.** 1996. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. Mol Cell Biol **16:**5276-87.

73.   **Wang, L. M., A. Kuo, M. Alimandi, M. C. Veri, C. C. Lee, V. Kapoor, N. Ellmore, X. H. Chen, and J. H. Pierce.** 1998. ErbB2 expression increases the spectrum and potency of ligand-mediated signal transduction through ErbB4. Proc Natl Acad Sci U S A **95:**6809-14.

74.   **Webster, M. A., and W. J. Muller.** 1994. Mammary tumorigenesis and metastasis in transgenic mice. Semin Cancer Biol **5:**69-76.

75.   **Weiner, D. B., J. Liu, J. A. Cohen, W. V. Williams, and M. I. Greene.** 1989. A point mutation in the neu oncogene mimics ligand induction of receptor aggregation. Nature **339:**230-1.

76.   **Weiss, A., and J. Schlessinger.** 1998. Switching signals on or off by receptor dimerization. Cell **94:**277-80.

77.   **Xie, W., A. J. Paterson, E. Chin, L. M. Nabell, and J. E. Kudlow.** 1997. Targeted expression of a dominant negative epidermal growth factor receptor in the mammary gland of transgenic mice inhibits pubertal mammary duct development. Mol Endocrinol **11:**1766-81.

78.   **Yang, J., K. Symes, M. Mercola, and S. L. Schreiber.** 1998. Small-molecule control of insulin and PDGF receptor signaling and the role of membrane attachment. Curr Biol **8:**11-8.

79.   **Zhang, K., J. Sun, N. Liu, D. Wen, D. Chang, A. Thomason, and S. K. Yoshinaga.** 1996. Transformation of NIH 3T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2. J Biol Chem **271:**3884-90.

80.   **Zrihan-Licht, S., J. Lim, I. Keydar, M. X. Sliwkowski, J. E. Groopman, and H. Avraham.** 1997. Association of csk-homologous kinase (CHK) (formerly MATK) with HER- 2/ErbB-2 in breast cancer cells. J Biol Chem **272:**1856-63.

FIGURE 1

A

Bivalent ligand		Induced dimers	
AP1510			FKBP/FKBP
Rapamycin			FKBP/FRB

B

Xba 1 Spe 1
           Sto

p75.F1.HA   MSV 5LTR   FKBP HA

p75.F2.HA   MSV 5LTR

p75.R1.Flag   MSV 5LTR   FRB Flag

p75

C

ErbB chimera

p75 Extracellular domain

p75 Transmembrane domain

ErbB1 Intracellular domain

Kinase domain

Y 891
Y 920
Y 992
Y 1068
Y 1086
Y 1148
Y 1173
FKBP
HA

D

Homodimer   Heterodimer

B1/B1   B2/B1

FIGURE 2

A

AP1510   FK506

0 50 100 250 500 1000 100 1000 EGF

198—

← EGFR

← p75.B1.F1

*
*

75—

*
* } Shc
*

49—

*
*
*

1 2 3 4 5 6 7 8 9

Blot: anti-pTyr

B

1 2 3 4 5 6 7 8 9

IP: anti-HA   Blot: anti-pTyr

1 2 3 4 5 6 7 8 9

Blot: anti-EGFR

FIGURE 3

A

IP: NMS   anti-HA   Total lysate

AP1510 → 0 50 500 0 50 100 500

p75.B1.F1 →

1 2 3 4 5 6 7 8
Blot: anti-pTyr

1 2 3 4 5 6 7 8
Blot: anti-Grb2

1 2 3 4 5 6 7 8
Blot: anti-Shc

1 2 3 4 5 6 7 8
Blot: anti-EGFR

B

p75.B1.F1   Rat1

AP1510 → 0 250 500 1000 0 50 1000 EGF

1 2 3 4 5 6 7 8
Blot: anti-Erk2

48

FIGURE 4

A

p75.B1.F2   p75.B2.F2

0 100 500 EGF   0 100 500 EGF

197 —

← p75.B2.F2

p75.B1.F2 ➤

97 —

67 —

← Shc

1   2   3   4   5   6   7   8

Blot: anti-pTyr

B

1   2   3   4   5   6   7   8

Blot: anti-phospho-Akt

1   2   3   4   5   6   7   8

Blot: anti-Akt

C

1   2   3   4   5   6   7   8

Blot: anti-Erk2

D

p75.B1.F2   p75.B2.F2

AP1510 ➤ 0   50   250 1000   0   50   250 1000

p75.B1.F2 ➤

Cbl ➤

1   2   3   4   5   6   7   8

IP: anti-Cbl   Blot: anti-pTyr

1   2   3   4   5   6   7   8

Blot: anti-Cbl

49

A

p75.B1.F2

p75.B2.F2

EtOH   AP1510

EtOH   AP1510

Minutes → | 0 | 0 | 30' | 60' | 10' | 30' | 60' | 0 | 0 | 30' | 60' | 10' | 30' | 60'

215 —

← p75.B2.F2

p75.B1.F2 →

1 2 3 4 5 6 7 8 9 10 11 12 13 14

IP: NeutrAvidin   Blot: anti-HA

B

1 2 3 4 5 6 7 8 9 10 11 12 13 14

IP: NeutrAvidin   Blot: anti-β1

FIGURE 5

FIGURE 6

AP1510 (nM)

FIGURE 7

p75.F1

p75.B1.F2

p75.B2.F2

Trypsinized and replated

p75.B1.F1

FIGURE 8

53

FIGURE 9

A

p75.B2.F2.HA →
p75.B1.F2.HA →

← p75.kdB1.R1.Flag

1 2 3   4 5 6

Blot: anti-HA   anti-Flag

B

B1.F2.HA+   B2.F2.HA +
kdB1.R1.Flag   kdB1.R1.Flag

AP1510   Rap   AP1510   Rap

0 100 500 50 100 250   0 100 500 50 100 250

← p75.B2.F2.HA

p75.B1.F2.HA →

1 2 3 4 5 6 7 8 9 10 11 12

IP: anti-HA   Blot: anti-pTyr

1 2 3 4 5 6 7 8 9 10 11 12

IP: anti-HA   Blot: anti-HA

C

← p75.B2.F2.HA

p75.B1.F2.HA →
p75.kdB1.R1.Flag →

1 2 3 4 5 6 7 8 9 10 11 12

IP: anti-Flag   Blot: anti-pTyr

p75.kdB1.R1.Flag →

1 2 3 4 5 6 7 8 9 10 11 12

IP: anti-Flag   Blot: anti-Flag

54

FIGURE 10

p75.kdB1.R1.Flag

B1.F2.HA   B2.F2.HA   B1.F2.HA   B2.F2.HA

0 50 100   0 50 100   0 50 100   0 50 100 ← Rap

197 —

p75.B2.F2 →   → p75.B2.F2
p75.B1.F2 →   → p75.B1.F2
p75.kdB1.R1 →   → p75.kdB1.R1

                                                          → Cbl
97 —

1   2   3   4   5   6   7   8   9   10   11   12

IP: anti-Flag   IP: anti-Cbl

Blot: anti-pTyr

7   8   9   10   11   12

Blot: anti-Cbl

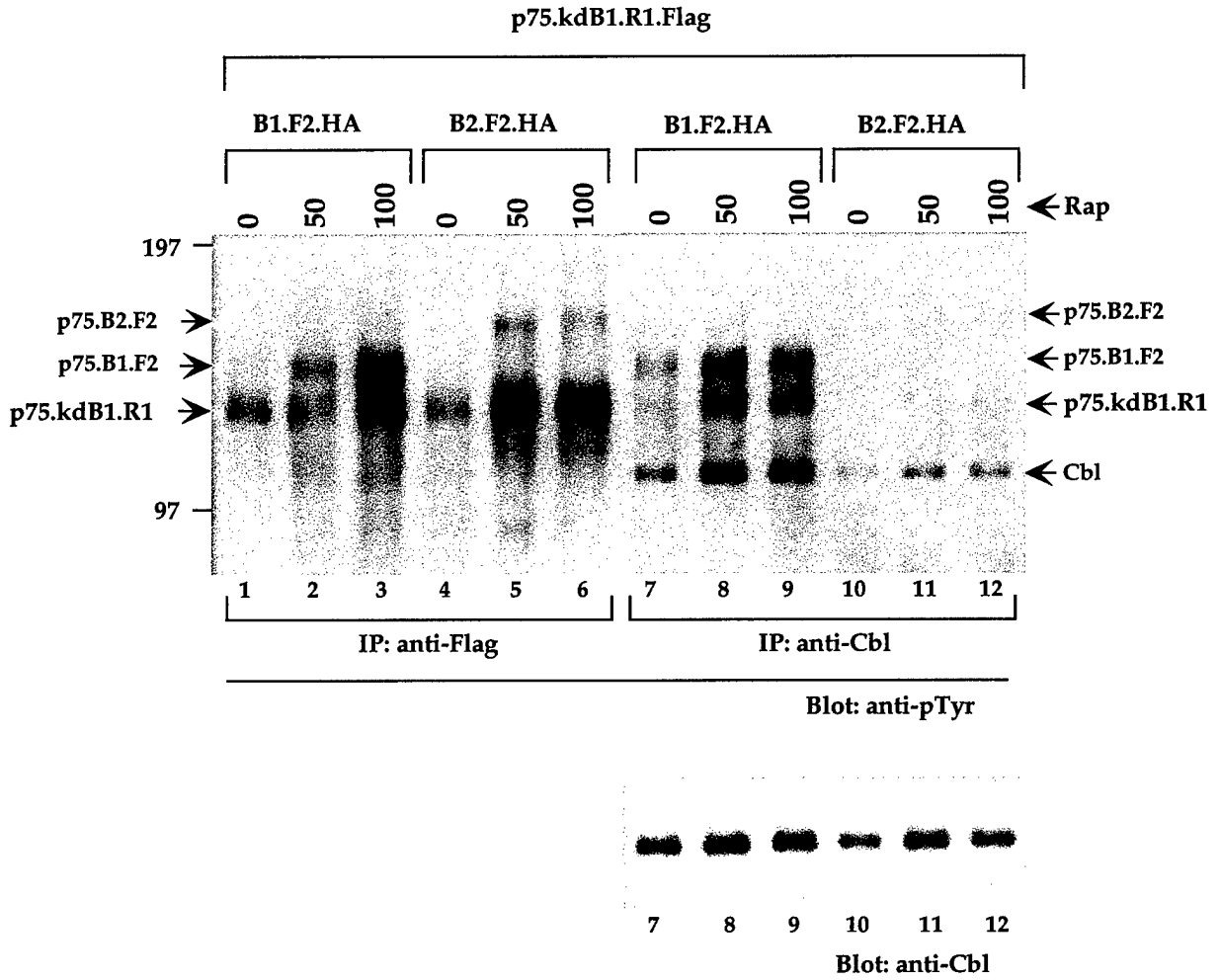# ErbB1 and ErbB2 differ in their ability to associate with and phosphorylate c-Cbl

FIGURE 11

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