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Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas

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In the present study we demonstrate that *erb*B-3 and *erb*B-2 cooperate in neoplastic transformation. Under conditions in which neither gene alone induced transformation, they readily transformed NIH3T3 cells if co-expressed. Furthermore, at high expression levels of ErbB2 which cause transformation, ErbB3 enhanced focus formation by one order of magnitude. Synergy required an intact ErbB2 extracellular domain and tyrosine kinase activity. Cooperation between ErbB3 and ErbB2 involved heterodimerization and increased tyrosine phosphorylation of ErbB3. Signaling by the heterodimer resulted in increased PI 3-kinase recruitment as well as quantitative and qualitative differences in substrate phosphorylation. Evidence for signaling by an active ErbB3-ErbB2 heterodimer in four mammary tumor cell lines indicated relevance of this mechanism for human neoplasia. Our detection of the NDF/heregulin transcript in NIH3T3 cells implicates an autocrine loop involving this ligand in signaling by the ErbB3-ErbB2 heterodimer in the model system, whereas heregulin-independent mechanisms likely exist for cooperative signaling by ErbB3 and ErbB2 chronically activated in some human mammary carcinomas.

Keywords: *erb*B receptor family; oncogenes; tyrosine phosphorylation; heterodimerization; PI3-kinase

Introduction

The *erb*B family of receptor tyrosine kinases (RTKs) includes the epidermal growth factor receptor (EGFR), *erb*B-2/HER-2/*neu*, *erb*B-3/HER-3 (Ullrich and Schlessinger, 1990 and references therein), and *erb*B-4/HER-4 (Plowman *et al.*, 1993). Members of this family have frequently been implicated in human neoplasia due to their overexpression in the presence or absence of gene amplification (Yokota *et al.*, 1986; Slamon *et al.*, 1987, 1989; Kraus *et al.*, 1987; van de Vijver *et al.*, 1987; Berger *et al.*, 1988). In the case of *erb*B-2, gene amplification in mammary and ovarian carcinomas has been associated with decreased disease-free survival (Slamon *et al.*, 1987, 1989). Understanding of the physiologic functions of these receptors has been greatly aided by studies in cell model systems in which they have been exogenously expressed individually or in combination. Such investigations have established, for instance, that ligand activation was indispensable for EGFR biological activity (Di Fiore *et al.*, 1987a; Velu *et al.*, 1987; Riedel *et al.*, 1988), while normal ErbB2 exhibited constitutive activity in the apparent absence of a specific ligand (Di Fiore *et al.*, 1987b; Hudziak *et al.*, 1987). Furthermore, structural receptor alterations were able to upregulate intrinsic catalytic properties of either EGFR or ErbB2 proteins (Di Fiore *et al.*, 1987b; Bargmann and Weinberg, 1988; Segatto *et al.*, 1988; Haley *et al.*, 1989). Finally, EGF-dependent phosphorylation of ErbB2 by the EGFR and biological cooperation of both molecules implicated transphosphorylation and heterodimerization in signal transduction by this class of receptors (King *et al.*, 1988; Stern and Kamps, 1988; Kokai *et al.*, 1989).

Employing a chimeric EGFR/ErbB3 receptor, we and others have demonstrated that a third member of this receptor family, ErbB3, is capable of transducing mitogenic signals in NIH3T3 fibroblasts (Kraus *et al.*, 1993; Fedi *et al.*, 1994; Prigent and Gullick, 1994). The mechanisms responsible for ErbB3-induced mitogenesis, however, are not clear. Concerns about the intrinsic enzymatic ability of ErbB3 arose from possibly inactivating amino acid substitutions in the predicted ErbB3 catalytic domain with respect to other RTKs (Kraus *et al.*, 1989; Plowman *et al.*, 1990). Our initial results showed that ErbB3 is capable of autophosphorylation *in vitro* (Kraus *et al.*, 1993). However, the ErbB3 enzymatic activity might be significantly lower than that of the EGFR, as was shown in subsequent studies (Guy *et al.*, 1994).

Recent evidence provides a framework for delineating the role of ErbB3 in mitogenesis. It was reported that the EGFR is capable of transphosphorylating ErbB3, an event associated with recruitment of phosphatidylinositol 3-kinase (PI 3-kinase) (Soltoff *et al.*, 1994). In addition, we found that a ligand-stimulated EGFR/ErbB3 receptor can efficiently couple to PI 3-kinase, but not to phospholipase Cγ (PLCγ) or GTPase-activating protein (GAP), revealing striking differences as compared to the EGFR and ErbB2 proteins (Fedi *et al.*, 1994). Moreover, members of the NDF/heregulin family of EGF-like molecules (Holmes *et al.*, 1992; Wen *et al.*, 1992; Falls *et al.*, 1993; Marchionni *et al.*, 1993) bind to ErbB3 (Carraway *et al.*, 1994). However, co-expression of ErbB2 and ErbB3 in COS cells indicated that a putative heterodimer between these molecules bound heregulin 10- to 100-fold more efficiently than ErbB3 alone (Sliwkowski *et al.*, 1994; Carraway and Cantley, 1994). It is therefore conceivable that NDF/heregulin and/or other putative

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ligands normally associate with ErbB3-containing heterodimers. In this setting, ErbB3 would contribute its unique ability when compared with EGFR and ErbB2, to recruit PI 3-kinase, and possibly other second messengers. The mitogenic ability of ErbB3 might therefore depend in part on an intrinsic, albeit reduced, kinase activity and in part on its ability to heterodimerize with other *erb*B family members which are often co-expressed in various cell types.

ErbB3 has been found to be expressed at varying levels in human mammary carcinomas (Kraus *et al.*, 1989; Lemoine *et al.*, 1992). Furthermore, chronic ErbB3 tyrosine phosphorylation in a significant fraction of such tumor cell lines indicated constitutive activation of the ErbB3 signaling function in these tumors (Kraus *et al.*, 1993). We reasoned that ErbB3 might cooperate with other *erb*B family members by simultaneous recruitment of complementary signal transduction pathways. In the present study we tested this hypothesis. We demonstrate that co-expression of ErbB3 and ErbB2 in NIH3T3 cells results in significantly increased transforming activity when compared to ErbB3 or ErbB2 expressed individually. Analysis of cytoplasmic signaling pathways indicated that cooperative transformation was associated with qualitative and quantitative differences in substrate phosphorylation, including a striking increase in PI 3-kinase recruitment. Simultaneous activation of ErbB3 and ErbB2 signaling functions at both receptor and substrate levels was observed in certain human breast tumor cell lines, underscoring the relevance of biological cooperation between ErbB2 and ErbB3 for human neoplasia. Finally, lack of heregulin expression in mammary tumor cell lines harboring concomitantly activated ErbB2 and ErbB3, raises the possibility that heregulin-independent mechanisms exist for cooperation of ErbB2 and ErbB3 in human neoplasia.

Results

Evidence for concomitant activation of ErbB3 and ErbB2 in human mammary tumor cell lines

We have previously demonstrated constitutive ErbB3 tyrosine phosphorylation in some human mammary tumor cell lines, including MDA-MB453, BT474, SK-BR-3 and MDA-MB175 (Kraus *et al.*, 1993 and Table 1). As the same cell lines have also been shown to overexpress ErbB2, but not the EGFR (Kraus *et al.*, 1987), we sought to determine the state of ErbB2 tyrosine phosphorylation in the same cell lines. As shown in Figure 1, the ErbB2 protein was detected at varying levels in 10 mammary tumor cell lines, with highest expression in MDA-MB453, BT474, MDA-MB361 and SK-BR-3 (Figure 1). When anti-ErbB2 immunoprecipitates were subjected to immunoblot analysis with anti-P-Tyr, ErbB2 tyrosine phosphorylation was readily detected in MDA-MB453, BT474, MDA-MB175 and SK-BR-3 (Figure 1). These findings coincided with the presence of constitutive ErbB3 phosphorylation in the same cell lines (Table 1). ErbB2 tyrosine phosphorylation was not solely a function of its level of expression as evident from a comparison of MDA-MB175 and MDA-MB361 cells (Figure 1). These findings indicated concomitant chronic tyrosine phosphorylation of ErbB3 and ErbB2 in human mammary tumor cell lines, raising the possibility that ErbB3 and ErbB2 might be active synergistically.

Table 1 Concomitant activation of ErbB3 and ErbB2 signaling in human mammary carcinomas

Mammary carcinoma cell lines	*ErbB3 Protein*	*ErbB3 P-Tyr*	*ErbB2 Protein*	*ErbB2 P-Tyr*
MDA-MB453	++	++	++	+
BT474	++	++	+++	++
MDA-MB175	(+)	++	+	+
SK-BR-3	+	+	+++	++
MDA-MB134	++	–	(+)	–
MCF-7	+	–	(+)	–
MDA-MB361	++	–	++	–
MDA-MB415	+	–	(+)	–
ZR-75-1	+	–	+	–
MDA-MB468	++	–	(+)	–

Relative ErbB3 or ErbB2 protein and phosphotyrosine levels were determined by immunoblot analysis with protein-specific peptide antisera and anti-P-Tyr antibodies, following immunoprecipitation from 1 mg protein lysates with receptor-specific monoclonal antibodies. Relative signal intensities were evaluated semiquantitatively: +++/++/+ positive, (+) weakly positive, – not detectable. ErbB3 phosphotyrosine and protein levels have been previously reported (Kraus *et al.*, 1993)

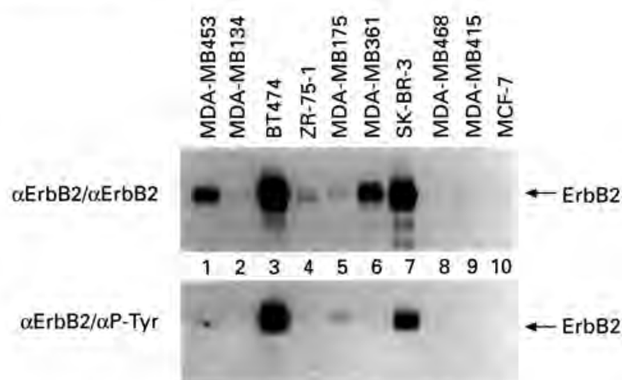

Figure 1 ErbB2 tyrosine phosphorylation in human mammary tumor cell lines. One mg lysate of the indicated mammary tumor cell lines prepared in SDS-containing buffer were immunoprecipitated with the ErbB2-specific monoclonal antibody E2-1. Immunoprecipitates were divided for immunoblot analysis with ErbB2 peptide antiserum M6 (Di Fiore *et al.*, 1990) or monoclonal anti-P-Tyr antibodies (PY20, PY69; ICN)

Cooperative transformation of NIH3T3 cells by ErbB2 and ErbB3

To investigate the possibility of cooperation between ErbB3 and ErbB2 in transformation, we cotransfected expression vectors for these proteins into NIH3T3 cells. In this model system, transformation by the normal ErbB2 protein has previously been shown to depend on its expression level (Di Fiore *et al.*, 1987b), whereas ErbB3 does not induce detectable focus formation (Kraus *et al.*, 1993). As shown in Figure 2, cotransfection with ErbB3 resulted in greatly enhanced ErbB2 transformation as compared to ErbB2 alone (Figure 2). LTR-ErbB3 cotransfection increased LTR-ErbB2 transforming ability by at least 10-fold, while it had no effect on EGFR-induced transformation, either in the presence or absence of EGF stimulation (Table 2). Strikingly, ErbB3 co-expression was able to unmask ErbB2 transforming ability at low levels of ErbB2 expression under SV40 transcriptional control. Con-

versely, ErbB3 did not synergize with an ATP-binding site mutant of ErbB2, LTR-ErbB2$_{R753}$ (Segatto *et al.*, 1990) or with an amino-terminally truncated ErbB2, LTR-ΔNErbB2 (Di Fiore *et al.*, 1987b), (Table 2) indicating a requirement for both intact ErbB2 tyrosine kinase activity and the presence of its extracellular domain.

Cooperative transformation by ErbB3 and ErbB2 is associated with increased ErbB3 phosphorylation and receptor heterodimerization

To investigate the effects of cotransfection on receptor activity, cotransfectants were marker selected and grown up to mass cultures. Following immunoprecipitation with ErbB3 or ErbB2-specific antibodies, immunoprecipitates were subjected to immunoblotting with anti-receptor or anti-P-Tyr. In an attempt to avoid receptor coprecipitation, lysates were prepared in the presence of ionic detergents. As shown in Figure 3A, ErbB3 was expressed at comparable levels in LTR-ErbB3 transfectants and cotransfectants with either LTR-ErbB2 or LTR-ErbB2$_{R753}$ (lane 7, 9 and 10), while endogenous mouse ErbB3 and ErbB2 proteins were not detectable (lanes 1 and 6). Expression levels of the normal ErbB2 protein were similar in cotransfectants with a control vector or with LTR-ErbB3 (lanes 3 and 4). By comparison, the ErbB2 kinase-negative mutant protein was expressed at about threefold lower levels in cotransfectants with LTR-ErbB3 (lane 5).

Analysis of ErbB2 immunoprecipitates with anti-P-Tyr antibodies demonstrated similar levels of ErbB2-specific tyrosine phosphorylation in cotransfectants with LTR-neo or LTR-ErbB3 (lanes 3 and 4), while no ErbB2 tyrosine phosphorylation was detectable in ErbB2$_{R753}$ mutant co-expressed with the ErbB3 protein (lane 5). These findings indicated that ErbB3 did not noticeably affect ErbB2 tyrosine phosphorylation. In

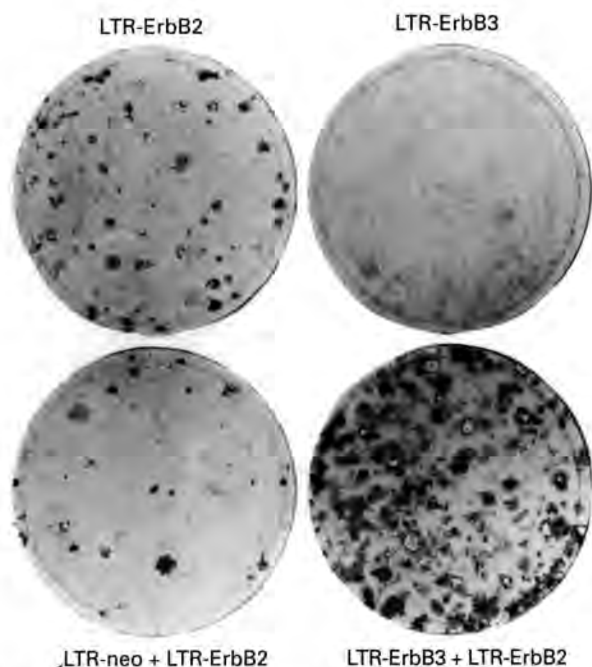

Figure 2 Biological phenotype of NIH3T3 transfectants. LTR-neo (1 μg), LTR-ErbB3 (1 μg) and LTR-ErbB2 (0.01 μg) expression vectors were transfected by calcium phosphate precipitation. Plates were stained with GIEMSA 14 days following transfection

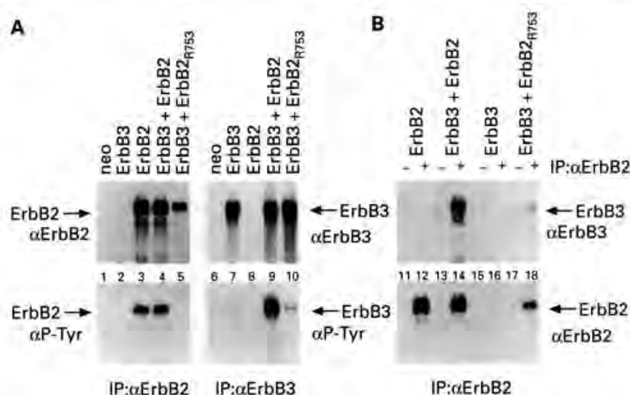

Figure 3 ErbB3 transphosphorylation and heterodimerization with ErbB2. Selected mass cultures of LTR-neo (neo) and LTR-ErbB3 (ErbB3) transfectants, or cotransfectants LTR-ErbB2/LTR-neo (ErbB2), LTR-ErbB3/LTR-ErbB2 (ErbB3 + ErbB2) and LTR-ErbB3/LTR-ErbB2$_{R753}$ (ErbB3 + ErbB2$_{R753}$) were serum-starved for 48 h and lysed in the presence of phosphatase inhibitors. (**A**) One mg lysate was immunoprecipitated (IP) in the presence of ionic detergents with αErbB2 peptide antiserum M6 or αErbB3 monoclonal antibody E3-1 (Kraus *et al.*, 1993). Immunoprecipitates were divided for immunoblot analysis with αErbB2 (M6) and αErbB3 (Kraus *et al.*, 1993) peptide antisera or monoclonal αP-Tyr antibody. Direct immunoblot analysis confirmed relative ErbB3 and ErbB2 expression levels in these transfectants (data not shown). Furthermore, comparison with previously characterized transfectants (Di Fiore *et al.*, 1987b; Kraus *et al.*, 1993) indicated ErbB3 and ErbB2 receptor numbers of $\sim 1 \times 10^5$ and $\sim 2 \times 10^5$, respectively, whereas ErbB2$_{R753}$ was expressed at $\sim 7 \times 10^4$ molecules per cell. (**B**) Four mg fresh lysates prepared in the absence of ionic detergents were immunoprecipitated with αErbB2 monoclonal antibody E2-1 (+) or a non-immune control MOPC21 (−). Four fifth and 1/5 of immunoprecipitates were subjected to immunoblot analysis with αErbB3 and αErbB2 peptide antisera, respectively

Table 2 Biological cooperation of ErbB3 and ErbB2 in NIH3T3 cells

	LTR-neo (0.1)		*LTR-ErbB3 (0.1)*	
	FFU/plate	*CFU/plate*	*FFU/plate*	*CFU/plate*
SV40-ErbB2 (1)	<1	NT	22 (2)	NT
LTR-ErbB2 (0.01)	16 (4)	76 (13)	104 (10)	26 (6)
LTR-ErbB2$_{R753}$ (0.01)	<1	63 (12)	<1	12 (4)
LTR-ΔNErbB2 (0.01)	30 (4)	8 (2)	32 (6)	2 (1)
LTR-EGFR (0.1)	<1	79 (29)	1 (0.3)	35 (10)
LTR-EGFR (0.1) + EGF	13 (3)	NT	12 (4)	NT

NIH3T3 cells were transfected by the calcium phosphate precipitation method using 40 μg of calf thymus high molecular weight DNA as a carrier. FFU (standard error): Focus-forming units. CFU (standard error): Number of colonies exhibiting simultaneous resistance to G418 as well as kHAT, indicate stable transfection of both constructs. LTR-neo and LTR-ErbB3 vectors confer G418 resistance, where as LTR-driven ErbB2 and EGFR constructs carry the Ecogpt marker. Amounts (μg) of expression vector DNA transfected are listed in parentheses. Data were obtained from at least two independent transfection assays performed in duplicate

striking contrast, the level of ErbB3 tyrosine phosphorylation was elevated five- to 10-fold when co-expressed specifically with the normal ErbB2 protein (lane 7 and 9). There was no effect of co-expression with the ErbB2 ATP-binding site mutant (lanes 7 and 10) nor the activated ErbB2 protein (ΔNErbB2) that lacked most of its extracellular domain (data not shown). Thus, cooperative transformation of ErbB3 and ErbB2 was associated with a significant increase in ErbB3 tyrosine phosphorylation, suggesting transphosphorylation of ErbB3 by the ErbB2 protein.

We next sought to determine whether heterodimers between ErbB3 and ErbB2 could be demonstrated in the cotransfectants by testing the ability of ErbB2 to co-immunoprecipitate the ErbB3 protein. To minimize dissociation of receptor complexes, immunoprecipitation experiments were conducted on fresh lysates prepared in the absence of ionic detergents. Lysates were subjected to immunoprecipitation with an ErbB2-specific monoclonal antibody and then immunoblotted with peptide antibodies specific for ErbB3 or ErbB2 proteins, respectively. As shown in Figure 3B, the ErbB3 protein was readily recovered from ErbB2 immunoprecipitates of LTR-ErbB3/LTR-ErbB2 co-transfectants (lane 14). The specificity of this co-immunoprecipitation was confirmed by the absence of the ErbB3 protein in immunoprecipitates of the same lysates using non-immune IgG, or in ErbB2 immunoprecipitates of LTR-ErbB3 transfectants (Figure 3B, lanes 13 and 16). These results demonstrated that ErbB3 and ErbB2 aggregate *in vivo* to form heterodimeric receptor complexes. Of note, heterodimer formation occurred independently of ErbB3 or ErbB2 tyrosine phosphorylation, since quantitatively proportional ErbB3 protein was co-immunoprecipitated with anti-ErbB2 from cells co-expressing ErbB3 and the ErbB2 ATP-binding site mutant protein (Figure 3B, lane 18).

ErbB3-mediated recruitment of PI 3-kinase activity is enhanced by co-expression of ErbB2

In efforts to investigate whether the biological cooperation observed between ErbB3 and ErbB2 was associated with alterations in their intracellular signaling capacities, we initially investigated PI 3-kinase activity in the cotransfectants. As shown in Figure 4, significantly elevated levels of PI 3-kinase activity were observed in anti-P-Tyr or anti-ErbB3 immunoprecipitates of LTR-ErbB3/LTR-ErbB2 cotransfectants when compared to LTR-neo or LTR-ErbB3 as well as LTR-neo/LTR-ErbB2 or LTR-ErbB3/LTR-ErbB2$_{R753}$ co-transfectants (Figure 4A). Standardization of the PIP signal relative to p85 expression levels in the same lysates indicated a 2–5-fold increase of PI 3-kinase activity. Thus, the increase in PI 3-kinase recruitment paralleled the increased level of ErbB3 tyrosine phosphorylation observed in LTR-ErbB3/LTR-ErbB2 cotransfectants.

We next compared association of the p85 subunit of PI 3-kinase with the fraction of phosphotyrosine-containing proteins in these transfectants. Immunoblot analysis with anti-p85 demonstrated that the highest levels of p85 were detected in anti-P-Tyr immunoprecipitates of ErbB3/ErbB2 cotransfectants (Figure 4B, lane 4). Relative to p85 expression levels in the same lysates, a 2.8-fold or 5.5-fold increase was estimated in these cotransfectants when compared to transfectants

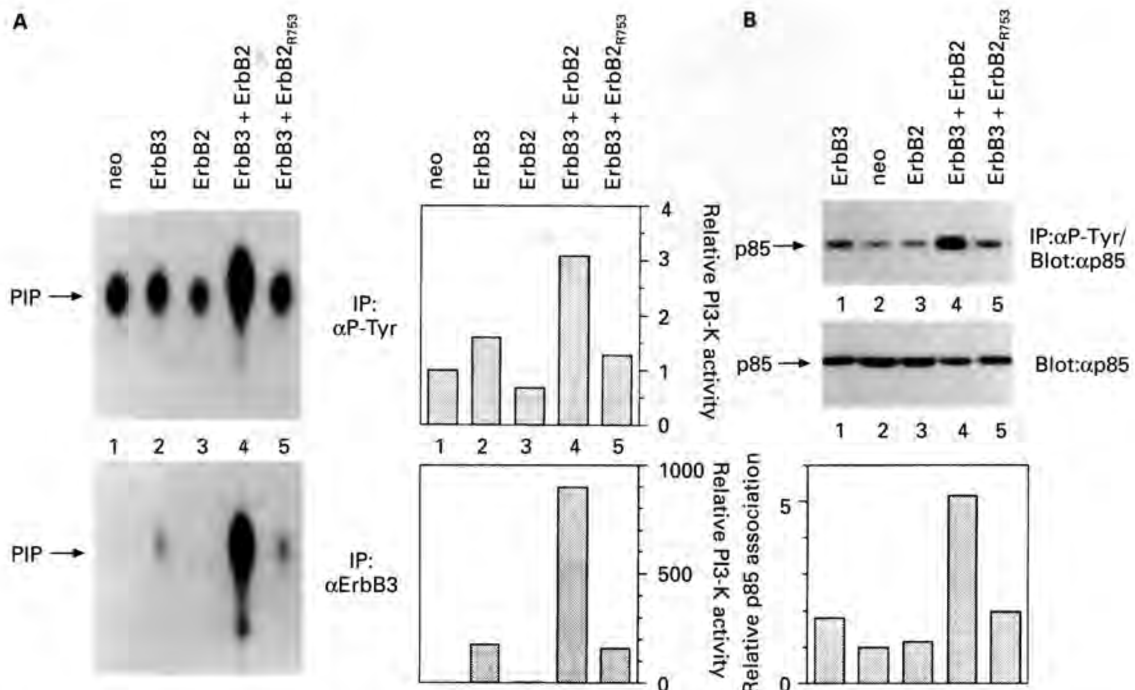

Figure 4 Enhanced signaling through PI 3-kinase by ErbB3-ErbB2 heterodimers. Two mg fresh lysates were immunoprecipitated with αP-Tyr or αErbB3 monoclonal antibodies and divided for PI 3-kinase assay (**A**) or immunoblot analysis with an αp85 antibody (UBI), (**B**). 100 μg of the same lysates were subjected to direct immunoblotting with αp85 (**B**). PIP (phosphatidylinositol 3-phosphate) and p85 signal intensities were determined by phosphor-imager analysis. Relative PI 3-kinase activity = PIP signal intensity/p85 protein. Relative p85 association = p85 signal intensity from αP-Tyr immunoprecipitates/p85 protein

containing only ErbB3 or ErbB2, respectively. Thus, heterodimerization of ErbB3 and ErbB2 was associated with a significant increase of PI 3-kinase signaling in NIH3T3 cells.

Cooperative transformation by ErbB3 and ErbB2 involves differences in substrate phosphorylation by receptor heterodimers

To explore whether other differences in substrate recruitment or phosphorylation by an ErbB2-ErbB3 heterodimer could be demonstrated, we sought to compare patterns of phosphotyrosine-containing proteins in transfectants expressing similar levels of ErbB3, ErbB2 or both proteins by two-dimensional gel analysis. As shown in Figure 5, phosphotyrosine-containing proteins were less abundant in LTR-ErbB3 as compared with ErbB2 transfectants. Comparison of cotransfectants expressing ErbB3 as well as ErbB2 with an ErbB2 control revealed a number of common spots. Some of these P-Tyr-containing proteins showed increased intensity in the cotransfectants and were detectable at lower intensity in the LTR-ErbB3 transfectant (Figure 5). These findings suggested a quantitative increase of substrate phosphorylation in LTR-ErbB3/LTR-ErbB2 cotransfectants. Of note, three major spots were uniquely detected in LTR-ErbB3/LTR-ErbB2 cotransfectants (Figure 5, right panel, arrowheads). Two of these proteins migrated in the molecular weight range of 120–130 kDa, whereas the third exhibited an estimated molecular mass of 65 kDa. None of these spots was visualized using antisera specific for carboxyl-terminal epitopes of ErbB2 or ErbB3 (data not shown), suggesting that they are not receptor degradation products but rather reflect tyrosine kinase substrates specifically phosphorylated by heterodimeric ErbB2-ErbB3 receptors. Two spots observed in the ErbB2 transfectant were not evident in the ErbB2/ErbB3 cotransfectant. It is not known whether the 65 kDa protein in the latter represents a differentially phosphorylated isoform of one of these two unique spots in the ErbB2 transfectants. In any case, these findings provide evidence for qualitative as well as quantitative differences in substrate phosphorylation in NIH3T3 cells cooperatively transformed by ErbB2 and ErbB3.

Evidence for ErbB3-mediated signaling in human breast tumor cell lines

Based upon the known differences in efficiency of ErbB3 and ErbB2 with respect to PI 3-kinase recruitment, we investigated the pattern of PI 3-kinase activation in six human breast tumor cell lines including four lines that exhibited active ErbB3 and ErbB2. PI 3-kinase activity was determined following serum-starvation and was normalized for p85 expression levels in these cell lines. Analysis of anti-P-Tyr and anti-ErbB3 immunoprecipitates revealed an increase in associated PI 3-kinase activity in MDA-MB361, MDA-MB453, MDA-MB175, BT474 and SK-BR-3 as compared to MDA-MB134 cells which showed little if any associated PI 3-kinase activity. Moreover, analysis of anti-ErbB3 immunoprecipitates revealed relatively higher levels of associated PI 3-kinase activity in cell lines exhibiting chronic ErbB3 and ErbB2 tyrosine phosphorylation (Figure 6A). All of these findings suggested increased coupling of ErbB3 with PI 3-kinase in the ErbB3 activated tumor cell lines.

We next analysed these same tumor cell lines for evidence of ErbB3-ErbB2 heterodimers. As shown in Figure 6B, ErbB3 co-immunoprecipitation with ErbB2 was most pronounced in the four cell lines with constitutively activated ErbB3 including MDA-MB453, BT474, SK-BR-3 and MDA-MB-175, whereas little co-immunoprecipitation was observed in MDA-MB361 cells. The extent of ErbB3 co-immunoprecipitation with ErbB2 in these cell lines corresponded to relative levels of chronic ErbB3 tyrosine phosphorylation as well as ErbB3-associated PI 3-kinase activity. Thus, the results with human breast tumor lines are consistent with heterodimer formation and PI 3-kinase recruitment observed in NIH3T3 cells cooperatively transformed by ErbB2 and ErbB3.

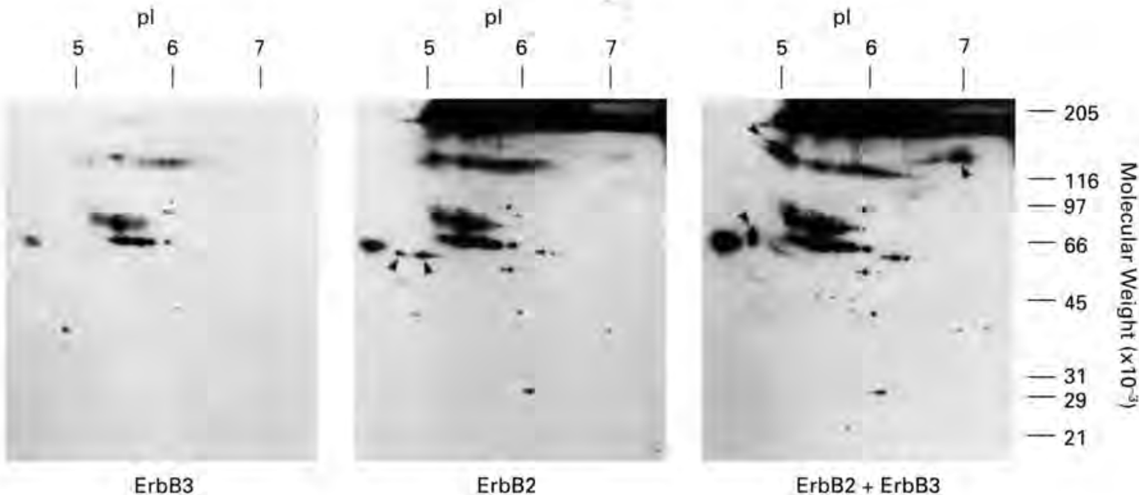

Figure 5 Two-dimensional analysis of phosphotyrosine-containing proteins in NIH3T3 transfectants. Serum-starved NIH3T3 transfectants were lysed in 1% Triton buffer containing phosphatase inhibitors. Six mg of total proteins were immunoprecipitated with excess of αP-Tyr antibody (UBI) and processed for 2-D analysis as described (Romano *et al*., 1994). Following electrotransfer phosphotyrosine-containing proteins were visualized with αP-Tyr antibody (UBI)

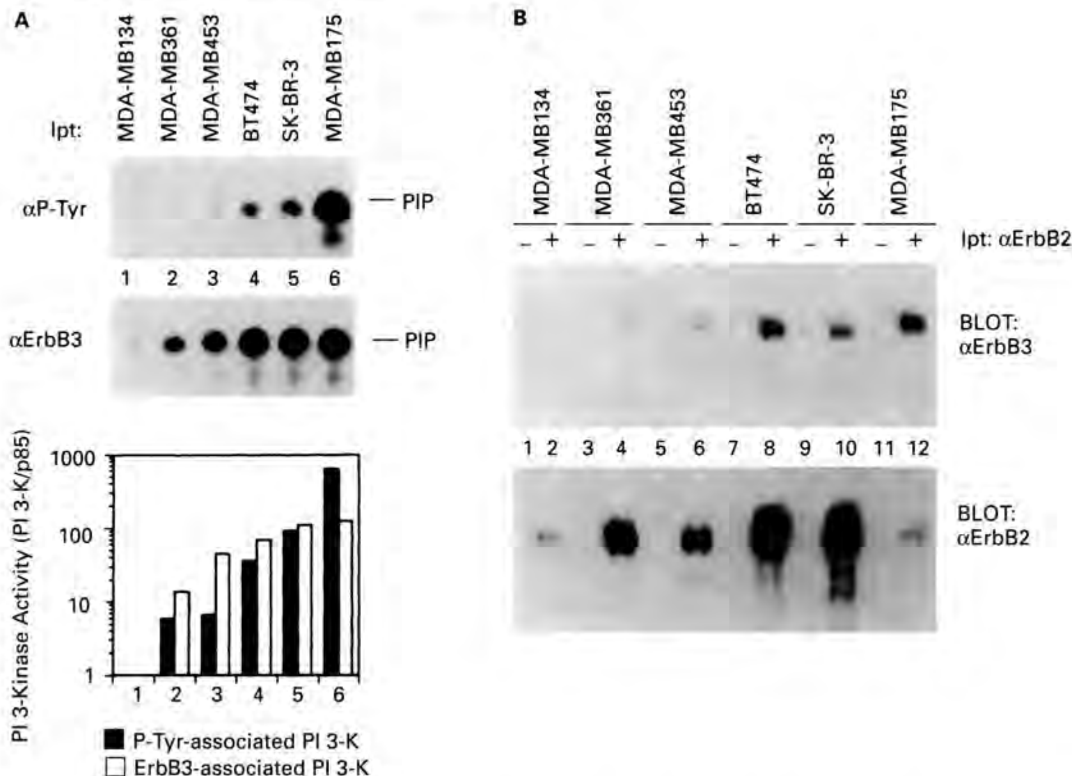

Figure 6 Signaling by an ErbB3-ErbB2 heterodimer in human mammary tumor cell lines. (**A**) PI 3-kinase activity associated with αP-Tyr or αErbB3 immunoprecipitates was determined as described in legend to Figure 4. (**B**) ErbB3-ErbB2 heterodimer formation was analysed in the indicated mammary tumor cell lines as described in Figure 3B

Ligand-dependent activation of ErbB3-ErbB2 heterodimers

Co-expression of ErbB2 and ErbB3 has been reported to reconstitute a high-affinity receptor for NDF/heregulin (Carraway and Cantley, 1994; Sliwkowski *et al.*, 1994). Thus, NDF/heregulin might be involved in ErbB3-ErbB2 heterodimer formation in NIH3T3 cells as well as breast tumor cell lines co-expressing these molecules. We tested these cell lines for NDF/heregulin transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR). Oligonucleotide primers were designed in invariant regions of the gene to allow PCR amplification of the rodent homologue (NDF) as well as 4 human isoforms of heregulin, HRG-α, HRG-β1, HRG-β2 and HRG-β3. As shown in Figure 7A, the predicted 567 bp NDF/heregulin fragment was amplified from RNA of NIH3T3 and a human leiomyosarcoma derived cell line, SK-LMS-1, which served as a positive control. Under these same conditions, the NDF/heregulin transcript was not detectable in MDA-MB134, MDA-MB361, SK-BR-3, MDA-MB175, BT474 or MDA-MB453. A *β*-actin control demonstrated integrity of the mRNA in each cell line (Figure 7A). The expression of NDF/heregulin transcript in NIH3T3 cells is consistent with the concept that this ligand is responsible for autocrine triggering of ErbB3-ErbB2 heterodimers in these cells.

To investigate the possibility of other ligands responsible for ErbB2-ErbB3 heterodimerization in mammary tumor cell lines, we tested conditioned medium for the ability to induce ErbB3 tyrosine phosphorylation in MDA-MB134 cells. This cell line expresses relatively high ErbB3 and low ErbB2 protein levels neither of which exhibits detectable tyrosine phosphorylation (Figure 1 and Kraus *et al.*, 1993). When serum-starved MDA-MB134 were exposed to conditioned medium prepared from breast tumor cell lines with active ErbB2 and ErbB3, conditioned medium of MDA-MB175 induced ErbB3 tyrosine phosphorylation, whereas SK-BR-3, BT474 and MDA-MB453 lacked similar activity. Thus, endogenous ligand production might be responsible for activated signaling by an ErbB3-ErbB2 heterodimer in MDA-MB175 cells. Whether this activity represents an alternatively spliced form of NDF/heregulin whose transcript was not detectable by RT-PCR or a different molecule, is not yet known.

Discussion

ErbB2 has been implicated in human malignancies due to gene amplification/overexpression and its ability to induce transformation in model systems *in vitro* and *in vivo*. In NIH3T3 cells, normal ErbB2 transforming activity was only evident at high levels of expression (Di Fiore *et al.*, 1987b; Hudziak *et al.*, 1987). In human malignancy, similarly high ErbB2 protein levels are observed in 20–30% of breast or ovarian carcinomas and are invariably associated with gene amplification (Yokota *et al.*, 1986; Kraus *et al.*, 1987; Slamon *et al.*, 1987; van de Vijver *et al.*, 1987; Berger *et al.*, 1988). However, a similar fraction of breast tumors and tumor-derived cell lines does not display gene amplification, but overexpresses ErbB2 at moderate levels, insufficient to produce the malignant phenotype in the NIH3T3 model system (Di Fiore *et al.*, 1987b;

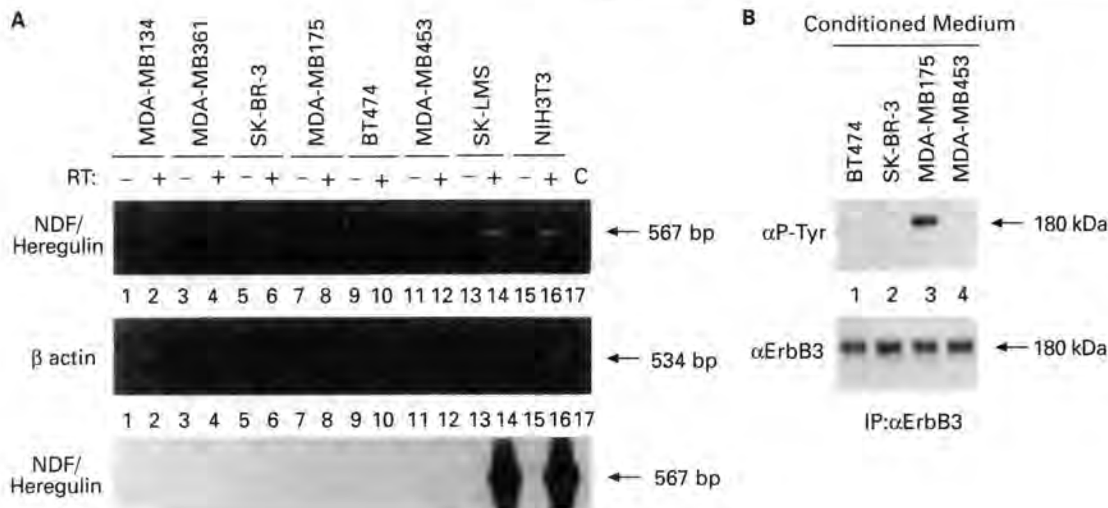

Figure 7 Ligand involvement in ErbB2-ErbB3 heterodimer activation. (**A**) Five μg total RNA from the indicated cell lines were analysed for the presence of NDF/heregulin transcript by RT-PCR. To ascertain RNA-specific amplification, reactions were performed in the presence or absence of reverse transcriptase (RT). In addition, a negative control for PCR amplification was conducted in the absence of template (C). Top: Ethidium bromide stain of NDF/heregulin products. Center: Ethidium bromide stain of β actin products. Bottom: Southern blot analysis of gel shown in top panel employing an internal NDF/heregulin-oligonucleotide as a probe. The predicted 567 bp fragment was independently confirmed as NDF/heregulin by restriction analysis with PstI and BclI. (**B**) Serum-starved MDA-MB134 cells were triggered with conditioned medium harvested from the indicated cell lines. Two mg lysate was immunoprecipitated with αErbB3 monoclonal antibody E3-1 and subjected to immunoblot analysis with αP-Tyr antibody (ICN) or αErbB3 peptide antiserum

Kraus *et al*., 1987; Berger *et al*., 1988; Slamon *et al*., 1989). Our present studies show that ErbB3 cooperates with ErbB2 in the neoplastic transformation of NIH3T3 cells. ErbB3 activates ErbB2 transforming potential at intermediate expression levels and substantially enhances neoplastic transformation, when ErbB2 is expressed at high protein levels. These observations in a model system were paralleled by evidence of concurrent activation of ErbB2 and ErbB3 signaling function in a number of human breast tumor cell lines. These results imply that biological cooperation between these two *erb*B family members can occur in human malignancy.

We demonstrated that biological cooperation between ErbB2 and ErbB3 was associated with heterodimer formation and increased steady state ErbB3 tyrosine phosphorylation. The formation of a heterodimer *in vivo* may facilitate transphosphorylation of ErbB3 by ErbB2 or such heterodimers may possess higher intrinsic catalytic activity than the monomer. We further showed that an ErbB2 mutant lacking most of its extracellular domain, ΔNErbB2, was unable to cooperate for transformation with ErbB3. Based upon the current understanding of receptor dimerization (Schlessinger, 1988), such a mutant would not display heterodimerization properties with ErbB3. This possibility was consistent with our finding that ErbB3 does not display increased tyrosine phosphorylation in ΔNErbB2/ErbB3 cotransfectants (data not shown). Heterodimer formation between the EGFR and ErbB2 has previously been reported to be associated with a cooperative transforming activity of these molecules (Kokai *et al*., 1989). However, constitutive EGFR-ErbB2 heterodimers were not reported in human tumor cells due to a lack of co-expression of these two receptors at sufficient protein levels (Goldman *et al*., 1990). Conversely, ErbB3 and ErbB2 are found simultaneously expressed in several human mammary tumor cell lines at intermediate or high protein levels. Moreover, an active ErbB3-ErbB2 heterodimer in MDA-MB175 suggests that simultaneously increased signaling can occur even at lower expression of both receptors.

The question arises as to the underlying mechanisms of ErbB2 and ErbB3 heterodimer formation. Our observations suggest involvement of multiple molecular mechanisms. Previous studies have indicated that receptor homodimerization and heterodimerization occur in response to ligand or, as in the case of ErbB2, may result from structural alterations mimicking ligand activation (Weiner *et al*., 1989; Honegger *et al*., 1990; Spivak-Kroizman *et al*., 1992). Based upon constitutive ErbB3 tyrosine phosphorylation in NIH3T3 cells, we have previously suggested that these cells may produce an ErbB3 ligand (Kraus *et al*., 1993). Consistent with this finding, we detected endogenous NDF/heregulin expression in NIH3T3 cells by RT-PCR. In addition, conditioned medium from NIH3T3 cells was able to trigger ErbB3 tyrosine phosphorylation in MDA-MB134 cells (data not shown). Thus, autocrine NDF/heregulin production is likely responsible for ErbB3-ErbB2 heterodimerization in this model system.

In all four mammary tumor cell lines displaying constitutive activation of ErbB2 and ErbB3, we failed to detect NDF/heregulin expression by RT-PCR. This observation does not rule out alternatively spliced NDF/heregulin transcripts escaping detection by RT-PCR. Such a NDF/heregulin variant might be present in MDA-MB175, since we observed soluble ErbB3-triggering activity as well as higher molecular weight transcripts hybridizing with a human heregulin probe in Northern blot analysis (data not shown). In SK-BR-3, MDA-MB453 and BT474, however, lack of NDF/heregulin detection by RT-PCR was paralleled by lack of transcript detection in Northern blot analysis (data

not shown and Holmes *et al.*, 1992) and absence of ErbB3 triggering activity from conditioned medium. These findings raise the possibility that ErbB2-ErbB3 heterodimerization in these cell lines involves a still unknown, cell-associated ligand activity or ligand-independent mechanisms including mutations or high receptor density.

We demonstrated enhanced signaling through the PI 3-kinase pathway in NIH3T3 cells as well as human tumor cell lines exhibiting co-expression of chronically activated ErbB2 and ErbB3. These findings can be explained by the enhanced tyrosine phosphorylation of ErbB3 co-expressed with ErbB2 which by itself does not efficiently couple with PI 3-kinase. Substantial evidence implicates PI 3-kinase in mitogenic signaling (Cantley *et al.*, 1991; Valius and Kazlauskas, 1993; Rodriguez-Viciana *et al.*, 1994). Thus, increased recruitment of PI 3-kinase might participate in enhanced signaling by an ErbB3-ErbB2 heterodimer. However, it appears unlikely that PI 3-kinase alone is responsible for this effect. SV40-ErbB2 transfected NIH3T3 cells, for instance, did not exhibit a transformed morphology even upon exposure to high concentrations of insulin, a potent activator of PI 3-kinase (Cantley *et al.*, 1991 and PP Di Fiore, unpublished observations), whereas they were readily transformed by co-expression of ErbB3. Indeed, we identified by 2-dimensional gel analysis three phosphotyrosine proteins that are uniquely phosphorylated in the cotransfectant, but not by ErbB3 or ErbB2 alone. Thus, biological cooperation does not only involve additive recruitment of ErbB3 and ErbB2 signaling pathways, but is determined by qualitative differences in substrate recruitment by a receptor heterodimer. Characterization of these substrate proteins should provide insight in the mechanism of action by an ErbB2-ErbB3 heterodimer.

In human breast cancer, ErbB2 overexpression associated with gene amplification has been shown to possess prognostic value for decreased survival in lymph-node positive disease, while a diagnostic role in stage I tumors was not unequivocally established (Slamon *et al.*, 1987, 1989; Tandon *et al.*, 1989; Paterson *et al.*, 1991). In addition, ErbB2 overexpression at intermediate levels affecting a significant subset of human mammary carcinomas, could not be associated with disease progression. Based on our results, it appears worthwhile to evaluate breast tumors for ErbB2 overexpression in light of simultaneous ErbB3 expression as well as ligands activating the signaling function of a heterodimer. Evidence for biological synergism of ErbB3 and ErbB2 in a model system and human breast tumor cell lines raises the possibility that determination of a simultaneously activated ErbB3 and ErbB2 signaling function may improve the predictability of disease-free survival in human breast cancer.

Materials and methods

Tumor cell lines

Human tumor cell lines were obtained from the American Type Culture Collection (ATCC) and were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum.

Preparation of conditioned medium and triggering of MDA-MB134

Confluent monolayers of cell lines seeded on fibronectin-coated plates were washed twice with PBS (phosphate-buffered saline) and exposed to 0.1 ml per cm^2 serum-free medium (DMEM containing 5 $\mu g\ ml^{-1}$ transferrin and 10^{-8} M selenium) for 48 h. Conditioned media were harvested and clarified at 10 000 *g*. MDA-MB134 mammary tumor cells were plated on fibronectin-coated plates and grown to confluence. Following serum starvation for 18h, monolayers were triggered with 0.1 ml per cm^2 of conditioned medium for 10 min at 37°C. Mock treatment with serum-free medium or conditioned medium of MDA-MB134 cells served as negative controls.

Generation of αErbB2 monoclonal antibody E2-1

A monoclonal antibody against the extracellular domain of ErbB2 was raised by immunization of Balb/c mice with live LTR-ErbB2 NIH3T3 transfectants as previously described (Kraus *et al.*, 1993). Hybridoma supernatants were screened by enzyme-linked immunosorbent assay (ELISA). Following double cloning by limiting dilution, supernatants were analysed by immunofluorescence and immunoprecipitation. One monoclonal antibody, MAb E2-1, was further characterized as IgG1 isotype. It specifically recognized the ErbB2 protein, and did not show cross-reactivity with EGFR, ErbB3 or ErbB4 proteins overexpressed in NIH3T3 cells.

Protein analysis

Immunoprecipitation and immunoblot analyses were conducted essentially as previously described (Kraus *et al.*, 1987, 1993). Immunoprecipitations using monoclonal antibodies were performed using Gammabind G agarose precoated with goat anti-mouse immunoglobulin G secondary antibody. For receptor co-immunoprecipitation, cells were serum-starved for 16 h and lysed in a buffer containing 20 mM Tris-HCl 7.5, 100 mM NaCl, 5 mM EGTA, 1% Nonidet P-40 (NP-40), protease and phosphatase inhibitors. Receptor-specific immunoprecipitations were performed in StaphA buffer (10 mM sodium phosphate pH 7.4, 100 mM NaCl, 5 mM EGTA, 1% Triton, 0.1% SDS, 0.5% deoxycholate), whereas immunoprecipitations with anti-P-Tyr (UBI) were conducted in 1% Triton buffer (Fedi *et al.*, 1994). PI 3-kinase assays (Fedi *et al.*, 1994) and 2-D analyses (Romano *et al.*, 1994) were performed as previously reported.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was conducted by standard methodology using a commercial preamplification system (Gibco-BRL). First strand-synthesis was primed from 5 μg total RNA using random hexamers (Pharmacia) and 200 units Superscript reverse transcriptase. Following inactivation of the enzyme at 95°C for 5 min, samples were treated for 20 min with 2.5 units of RNase H (Gibco-BRL). Forty cycles of PCR amplification (1 min at 95°C, 2 min at 55°C, 2 min at 72°C) were carried out in 100 μl using Taq polymerase (Boehringer Mannheim) and 1 μM of the following primers:

NDF/heregulin:

5′-GAGCGCAAAGAAGGCAGAGG-3′ and 5′-CACACA-GAAAGTTTTCTCCTT-3′ (Holmes *et al.*, 1992; Wen *et al.*, 1992).

β actin:

5′-TTCTACAATGAGCTGCGTGTG-3′ and 5′-CAGGA-AGGAAGGCTGGAAGA-3′ (Gunning *et al.*, 1983; Nudel *et al.*, 1983).

Southern blot hybridization

Following gel electrophoresis the NDF/heregulin RT-PCR products were transferred to nitrocellulose and hybridized with an internal NDF/heregulin-specific oligonucleotide matching NDF, HRGα and HRGβ1-3 (Wen *et al.*, 1992; Holmes *et al.*, 1992; 5′-CTTTGCACATATACTCTCCAG-3′), as previously described (Kraus and Aaronson, 1991) except using 8 × Denhardt's for hybridization. Stringencies during hybridization and washing were reduced by 28°C and 21°C, respectively (Kraus and Aaronson, 1991).

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