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Oncogenic forms of the *neu*/HER2 tyrosine kinase are permanently coupled to phospholipase Cγ

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The *neu*/HER2 proto-oncogene encodes a transmembrane tyrosine kinase homologous to receptors for polypeptide growth factors. The oncogenic potential of the presumed receptor is released through multiple genetic mechanisms including a specific point mutation, truncation at the extracellular domain and overexpression of the proto-oncogene. Here we show that all these modes of oncogenic activation result in a constitutively phosphorylated *neu* protein and an increase in tyrosine phosphorylation of a phosphatidylinositol-specific phospholipase (PLC$_\gamma$). The examined transforming *neu*/HER2 proteins, unlike the normal gene product, also co-immunoprecipitated with PLC$_\gamma$ molecules. A kinase-defective mutant of a transforming *neu* failed to mediate both tyrosine phosphorylation and association with PLC$_\gamma$, suggesting direct interaction of the *neu* kinase with PLC$_\gamma$. This possibility was examined by employing a chimeric protein composed of the extracellular ligand-binding domain of the epidermal growth factor receptor and the *neu* cytoplasmic portion. The chimeric receptor mediated rapid ligand-dependent modification of PLC$_\gamma$ on tyrosine residues. It also physically associated, in a ligand-dependent manner, with the phosphoinositidase. Based on the presented results we suggest that the mechanism of cellular transformation by the *neu*/HER2 receptor involves tyrosine phosphorylation and activation of PLC$_\gamma$.

Key words: growth factor receptors/phosphoinositides/protein phosphorylation/signal transduction

Introduction

The *neu* oncogene was originally identified as the transforming gene in a chemically transformed rat neuroblastoma cell line (Schechter *et al.*, 1984). The human homologue of *neu*, *HER2*/c-*erbB2*, was identified by virtue of its high homology to the gene that codes for the epidermal growth factor (EGF) receptor (Coussens *et al.*, 1985; Yamamoto *et al.*, 1986). The gene codes for a 185 kd glycoprotein that spans the plasma membrane once; its cytoplasmic domain is a tyrosine-specific protein kinase (Akiyama *et al.*, 1986; Stern *et al.*, 1986). The extracellular domain probably functions in ligand binding, as inferred from its high relatedness to the corresponding part of the EGF receptor and the detection of a candidate ligand molecule (Yarden and Weinberg, 1989; Lupu *et al.*, 1990; Yarden and Peles, 1991).

Oncogenic activation of the rat *neu* gene involves a single point mutation that results in the substitution of a valine residue (Val664) at the predicted transmembrane stretch for a glutamic acid residue (Bargmann *et al.*, 1986). Alternatively, partial activation of the transforming potential of the proto-oncogene can be achieved by extensive deletions of non-catalytic sequences at the amino-terminal half or at the carboxy-terminal tail of the molecule (Di Fiore *et al.*, 1987; Bargmann and Weinberg, 1988a). The human variant of the gene, *HER2*/c-*erbB2* can be similarly activated by a point mutation (Segatto *et al.*, 1988; Suda *et al.*, 1990). Overexpression of either the human or the rat gene leads to transformation of NIH-3T3 fibroblasts (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987; Di Marco *et al.*, 1990). This observation is relevant to human adenocarcinomas in which the *HER2*/c-*erbB2* gene is frequently amplified and over-expressed (Yokota *et al.*, 1986; Gullick *et al.*, 1987; Van de Vijver *et al.*, 1987; Kraus *et al.*, 1987), and particularly to mammary and ovarian carcinomas that display statistically relevant correlation between overexpression of the gene and parameters used in breast cancer prognosis (Slamon *et al.*, 1987; Varley *et al.*, 1987; Venter *et al.*, 1987; Zhou *et al.*, 1987; Berger *et al.*, 1988; Slamon *et al.*, 1989).

To examine the biochemical pathways elicited by activation of the *neu* receptor by its presumptive ligand, we generated monoclonal antibodies which, upon binding to p185^{neu}, activated its endogenous tyrosine kinase activity in living cells (Yarden, 1990). Using these antibodies we found that the transforming mutant of p185^{neu} is functionally equivalent to an antibody-stimulated normal p185^{neu} as it displayed constitutive tyrosine phosphorylation and accelerated receptor turnover. These observations led us to hypothesize that *neu*-mediated transformation utilizes biochemical pathways normally employed, in a regulated manner, by the cognate ligand of p185^{neu}. Another approach to study the cellular function of the neu protein utilized chimeric neu–HER2 proteins which are stimulated by EGF as a heterologous ligand (Lee *et al.*, 1989; Lehvaslaiho *et al.*, 1989). Biochemical analysis of cells expressing the chimeric receptor indicated that the neu tyrosine kinase is coupled to calcium fluxes and rapid metabolism of phosphoinositides (Pandiella *et al.*, 1989). Considering the above described equivalence of a transforming neu protein and ligand-occupied receptor, this observation raised the possibility that oncogenic forms of the *neu*–HER2 protein interact with the phosphatidylinositol signalling pathway.

The hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) is known to produce two second messengers: inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Whereas the former induces the release of Ca^{2+} from intracellular membrane stores (Berridge and Irvine, 1989), the latter allosterically activates protein kinase C (Nishizuka, 1989). The enzymatic hydrolysis is catalysed by either isozyme of the family of phospholipase C enzymes (Rhee

et al., 1989). Only one species, PLC_γ, has been shown to undergo modification upon treatment of cells with mitogens such as EGF, fibroblast growth factor (FGF) and the platelet derived growth factor (PDGF) (Wahl *et al.*, 1988, 1989a; Meisenhelder *et al.*, 1989; Margolis *et al.*, 1989; Burgess *et al.*, 1990). Moreover, physical associations between PLC_γ and the corresponding ligand-stimulated receptor have been observed (Meisenhelder *et al.*, 1989; Margolis *et al.*, 1989; Kumjian *et al.*, 1989; Morrison *et al.*, 1990).

If oncogenic activation of the neu−HER2 receptor indeed functionally mimics ligand binding, then transforming variants of the protein may be coupled to phosphatidylinositol (PI) turnover through tyrosine phosphorylation of phospholipase C (Margolis *et al.*, 1990b; Nishibe *et al.*, 1990). Here we report that in cells expressing either oncogenic version of the neu−HER2 protein, unlike in cells that express the non-transforming forms, PLC_γ molecules are constitutively phosphorylated on tyrosine residues. Moreover, PLC_γ molecules of the transformed cells form a kinase-dependent complex with the mutation- or ligand-activated neu−HER2 receptor.

Results

Constitutive tyrosine phosphorylation of PLC_γ in cells transformed by a point-mutated neu gene

We speculated that the transforming protein encoded by the point-mutated *neu* oncogene represents a persistently active receptor that is constantly coupled to its cytoplasmic effector molecules. As the transforming protein is a hyperactive tyrosine kinase (Bargmann and Weinberg, 1988b; Yarden, 1990), its immediate target molecules are expected to be phosphorylated on tyrosine residues. To examine this possibility in relation to PLC_γ, we compared the state of tyrosine phosphorylation of the phosphoinositidase in transfected NIH-3T3 fibroblasts that express the normal neu protein (Val664, NE19 cells) or the transforming receptor (Glu664, B104-1-1 cells). It has been previously shown that the latter cells are fully transformed (Bargmann *et al.*, 1986). The transformed and the normal cell lines express comparable numbers of $p185^{neu}$ (at the range of $1-3 \times 10^5$ molecules/cell) and PLC_γ molecules (Figure 1A). To inhibit dephosphorylation of tyrosine residues, the cells were pre-incubated with vanadate (50 μM) prior to their analysis. Western blot analysis of $p185^{neu}$ and PLC_γ (145 kd protein) immunoprecipitates from the transformed cells revealed that both proteins were constitutively phosphorylated on tyrosine residues (Figure 1B). In contrast, in cells expressing the Val664 variant of $p185^{neu}$ or in the control untransfected fibroblasts (labelled NIH), neither protein was tyrosine phosphorylated (Figure 1B). Importantly, anti-PLC_γ immunoprecipitates from B104-1-1 cells included a co-immunoprecipitated 185 kd phosphoprotein. This protein was identified as $p185^{neu}$ based on a control Western blot of the PLC_γ immunoprecipitates with an antibody directed against $p185^{neu}$ (Figure 1B, right hand panel). We therefore concluded that the transforming point mutant of the neu protein physically associates with the PLC_γ molecules.

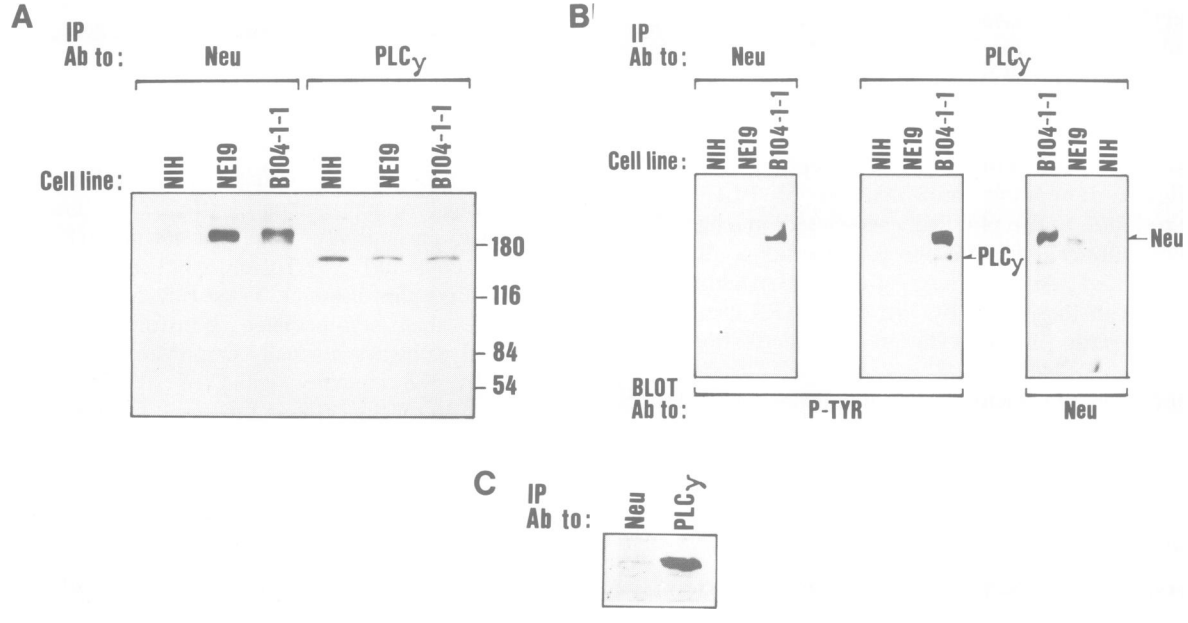

Fig. 1. Tyrosine phosphorylation of PLC_γ in fibroblasts expressing the normal and the oncogenic mutant of *neu−HER2*. Lysates were prepared from control untransfected NIH-3T3 fibroblasts (NIH), fibroblasts that overexpress the normal (Val664, NE19 cells) or the point mutant (Glu664, B104-1-1 cells) $p185^{neu}$. Cells were pre-incubated for 2 h at 37°C with Na_3VO_4 (50 μM) prior to cell lysis. Samples containing equal protein amounts ($\sim 3 \times 10^7$ cells) were then subjected to immunoprecipitation with either anti-PLC_γ antibodies (PC4 antiserum; 95% of the lysate), or anti-$p185^{neu}$ antibodies (NCT antiserum; 5% of the lysate) as indicated. (**A**) The cells were labelled with [^{35}S]methionine and then subjected to immunoprecipitation followed by gel electrophoresis and autoradiography. The locations of mol. wt marker proteins are shown in kd. (**B**) Unlabelled cells were analysed after immunoprecipitation with the antibodies indicated above the panels (IP Ab). The immunocomplexes were resolved by gel electrophoresis, transferred onto nitrocellulose filters and blotted with either antibodies to phosphotyrosine (PT5 antiserum) or antibodies to $p185^{neu}$ (NCT antiserum) as indicated below the panels. The blots were then probed with horseradish peroxidase-conjugated protein A followed by a chemiluminescence reagent as described under 'Materials and methods'. Autoradiograms (5 min exposure) are shown. The locations of PLC_γ and $p185^{neu}$ are indicated by arrows. (**C**) Stoichiometry of co-immunoprecipitation of PLC_γ with $p185^{neu}$. Lysates of B104-1-1 cells were subjected to immunoprecipitation with either antibodies to PLC_γ (10% of the lysate) or antibodies to $p185^{neu}$ (90% of the lysate). Following gel electrophoresis of the washed immunocomplexes and electrophoretic transfer onto nitrocellulose, the blots were probed with an antibody to PLC_γ.

Interestingly, PLC$_\gamma$ immunoprecipitates from cells expressing the normal neu protein contained relatively small but detectable amounts of p185^{neu} (Figure 1B, NE19 lane in the right hand panel). A relatively faint protein band of 140 kd was also detected in immunoprecipitates of p185^{neu} (Figure 1B). It underwent phosphorylation on tyrosine and co-immunoprecipitation with PLC$_\gamma$, but exhibited a slightly faster electrophoretic mobility than PLC$_\gamma$. By using different monoclonal and polyclonal antibodies, we identified this protein as a truncated form of p185^{neu}, which lacks part of the amino-terminal portion (unpublished results).

To estimate the fraction of the total cellular PLC$_\gamma$ that co-immunoprecipitated with the transforming p185^{neu}, an immunoprecipitate of the latter was blotted with antibodies to PLC$_\gamma$. Densitometric analysis indicated that 1% of PLC$_\gamma$ molecules physically associated with p185^{neu} in B104-1-1 cells (Figure 1C).

The effect of overall dephosphorylation of tyrosine phosphate was then examined by omitting the preincubation step with vanadate. As shown in Figure 2, the inclusion of vanadate significantly increased the level of tyrosine phosphorylation of PLC$_\gamma$, but it had almost no effect on the apparent amount of p185^{neu} that was associated with the enzyme (Figure 2, right panel). We therefore included vanadate in all our subsequent analyses.

PLC$_\gamma$ modification and complex formation with p185^{neu} are abolished in cells expressing a kinase-defective mutant of the oncogene

The transforming capacity of the *neu* oncogene has been previously shown to be absolutely dependent on the protein tyrosine kinase activity of the gene product (Bargmann and Weinberg, 1988a; Weiner *et al.*, 1989). To examine the dependency of the interaction of p185^{neu} with PLC$_\gamma$ on kinase activity, we introduced a mutation at the ATP-binding site of the transforming protein. This mutation replaced the essential lysine residue at position 758 with an alanine and resulted in a completely inactive kinase (Figure 3A) which also lost its transforming potential (data not shown). We next compared the state of tyrosine phosphorylation of PLC$_\gamma$ in cells that express the double mutant kinase defective p185^{neu} (Ala758, Glu664) with cells that express the single mutant transforming protein (Lys758, Glu664). The latter cell line (denoted by RB22) was established in parallel to the double mutant for appropriate controls. As shown in Figure 3B, these cell lines expressed similar levels of p185^{neu} and PLC$_\gamma$ (the 140 kd protein in neu immunoprecipitates is a degradation product of p185^{neu}). However, only in the RB22 cells were p185^{neu} and PLC$_\gamma$ constitutively phosphorylated on tyrosine residues and co-immunoprecipitated as a complex (Figure 3B). Hence, the interaction between p185^{neu} and PLC$_\gamma$ depends on the tyrosine kinase activity of the receptor.

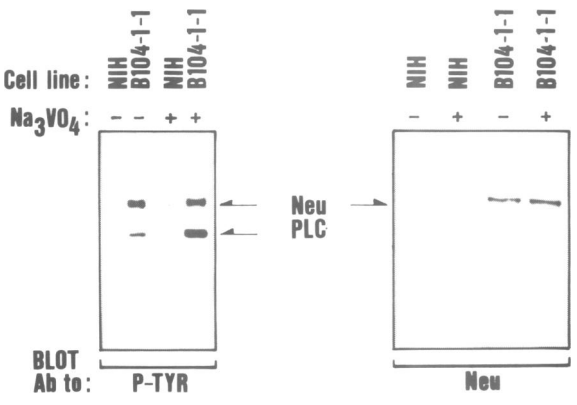

Fig. 2. Effect of vanadate pretreatment. The indicated cell lines in confluent 150 mm diameter dishes were treated for 2 h with or without Na_3VO_4 (50 μM) at 37°C and then subjected to immunoprecipitation with an antibody to PLC$_\gamma$, followed by Western blotting with either antibodies to phosphotyrosine (left panel) or to p185^{neu} (right panel).

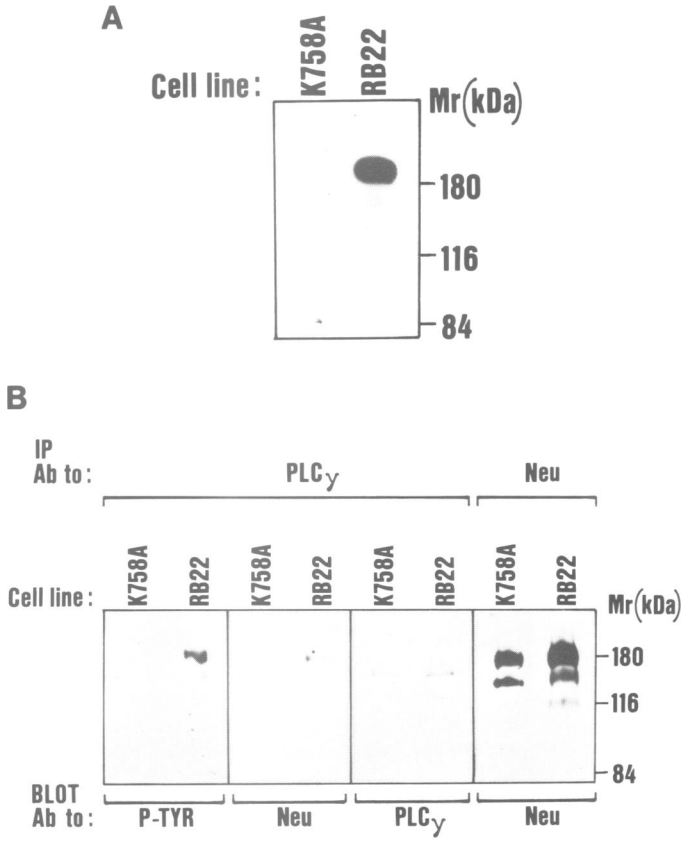

Fig. 3. Demonstration of lack of PLC$_\gamma$ modification in cells expressing a kinase defective mutant of the transforming p185^{neu}. Monolayers of cells expressing either the point-mutated transforming p185^{neu} (Glu664, RB22 cells), or a kinase defective mutant of it (Ala758, Glu664, K758A cells) were analysed. In the latter mutant, Lys758 of the ATP-binding site was replaced by an alanine residue. (**A**) *In vitro* kinase assay. One-tenth of each cellular lysate ($\sim 6 \times 10^7$ cells), containing the same amounts of total protein, was subjected to immunoprecipitation with a monoclonal antibody to p185^{neu} (43D6; Yarden, 1990). The washed immuno-complexes were incubated for 20 min at 22°C with 0.05 ml buffer containing 10 mM $MnCl_2$ and 5 μCi [γ-^{32}P]ATP (3000 Ci/mmol, Amersham). Following washes of the immunocomplexes they were resolved by gel electrophoresis and autoradiography (at −70°C with an intensifier screen). A 14 h exposure of the Kodak XAR film is shown and the locations of mol. wt marker proteins are indicated in kd. (**B**) Lysates prepared from 6×10^7 cells were aliquoted and immunoprecipitated with either anti-PLC$_\gamma$ or anti-p185^{neu} antibodies as indicated above the panels. The PLC$_\gamma$ immunoprecipitates were then divided into three parts, and all samples subjected to gel electrophoresis followed by transfer onto nitrocellulose filters. The filters were probed and detected as described in the legend to Figure 1. The antibodies used for blotting are indicated below the panel. The locations of prestrained mol. wt marker proteins (Sigma, St. Louis, MO) are indicated in kd. For the two right-hand panels, 5% of the cell lysates were used, whereas for the left pair of panels 45% of the lysate was used in each analysis.

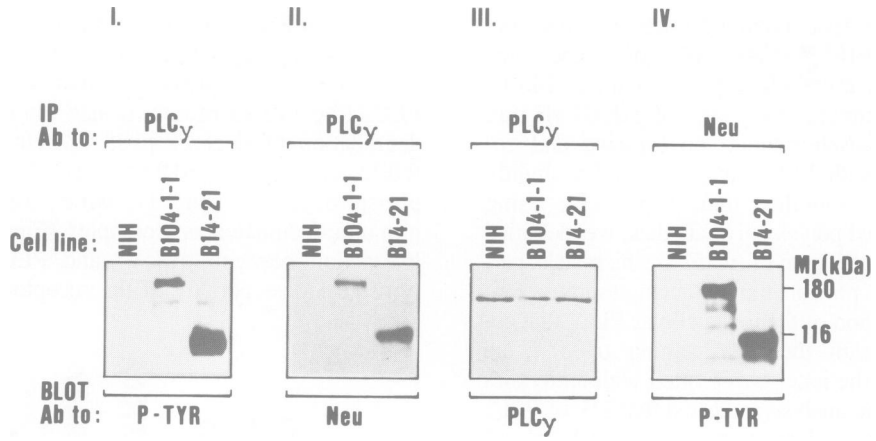

Fig. 4. Tyrosine phosphorylation and association of PLC_γ with an amino-terminally truncated mutant of $p185^{neu}$. The cell lines examined were control untransfected NIH-3T3 fibroblasts (NIH), the B104-1-1 cells expressing the Glu664 transforming mutant of $p185^{neu}$, and the B14-21 cells expressing an ectodomain-deficient Val664 form of the neu protein. Cell lysates containing equal amounts of total protein were subjected to immunoprecipitation with the antibodies indicated above the panels. Following gel electrophoresis, Western blotting was performed as described in the legend to Figure 1 with the antibodies indicated below each panel.

An oncogenic amino-terminal deletion mutant of neu also interacts with PLC_γ

To analyse further possible functional coupling between the oncogenic potential of neu−HER2 and the interaction with PLC_γ we examined an amino-terminally truncated form of the normal (Val664) protein. Deletion mutants of neu−HER2 that lack most of the extracellular domain have been previously shown to carry partial or full oncogenic potential (Bargmann and Weinberg, 1988a; Di Fiore *et al.*, 1987). We employed the B14-21 cell line which expresses a truncated rat *neu* gene (Bergmann and Weinberg, 1988a). Analysis of the truncated $p100^{neu}$ of these cells indicated that it underwent constitutive tyrosine phosphorylation in living cells (Figure 4, panel IV), suggesting that the deletion of the ectodomain releases a negative control over the kinase. In analogy with cells that express the Glu664 mutant of $p185^{neu}$, PLC_γ of the B14-21 cells exhibited a similar extent of elevated tyrosine phosphorylation (Figure 4, panel I). In addition, the truncated $p100^{neu}$, like the point-mutated $p185^{neu}$, associated physically with PLC_γ, as indicated by co-immunoprecipitation of both molecules after reaction with antibodies to PLC_γ (Figure 4, panel II).

PLC_γ and $p185^{HER2}$ are phosphorylated on tyrosine residues in cells that overexpress the HER2/c-erbB2 gene

Overexpression of either the human *HER2/c-erbB2* (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987) or the rat *neu* gene (Di Marco *et al.*, 1990) in mouse fibroblasts resulted in phenotypic transformation. Moderate overexpression of either gene was insufficient for cellular transformation (Hung *et al.*, 1986; Bargmann and Weinberg, 1988a; Di Fiore *et al.*, 1987). We attempted to test the possibility that this mode of oncogenic activation of the apparently normal receptor is associated with constitutive tyrosine phosphorylation of $p185^{HER2}$ and PLC_γ. To this end, we used mutant Chinese hamster ovary (CHO) cells which enable overexpression of genes that are introduced into them (Urlaub and Chasin, 1980). A series of CHO cell lines that express different numbers of the HER2/erbB2 protein was obtained through transfection and drug selection. These cell lines, denoted CER, expressed the human variant of *neu* in the range $1 \times 10^4 - 8 \times 10^5$ molecules per cell as determined by radioimmunoassay analysis with a monoclonal antibody to HER2/erbB2 (Figure 5A). When analyzed by Western blotting with antibodies to phosphotyrosine, the level of tyrosine phosphorylation of $p185^{HER2}$ was found to be proportional to the extent of overexpression (Figure 5B). The highest overexpressor, the CER50 cell line, displayed relatively high phosphorylation of both the intact as well as a smaller $p140^{HER2}$ protein. The latter protein was identified as a degradation product that lacks part of the extracellular domain and its appearance correlated with the extent of overexpression (Figure 5B and our unpublished results). Analysis of PLC_γ molecules of the CER50 line, in comparison with the other cell lines, revealed high tyrosine phosphorylation of the phosphoinositidase (Figure 5B). To examine physical association between the tyrosine phosphorylated $p185^{HER2}$ and PLC_γ molecules, immunoprecipitates of PLC_γ were probed with antibodies to neu−HER2 protein. As shown in Figure 5C, both forms of the overexpressed tyrosine kinase co-immunoprecipitated with PLC_γ molecules in CER50 cells.

This last observation of co-immunoprecipitation of the truncated $p140^{HER2}$ with PLC_γ could be explained in two ways: first, $p140^{HER2}$ may form heterodimers with the complete $p185^{HER2}$, or $p140^{HER2}$ may directly associate with PLC_γ, in analogy with $p100^{neu}$ (Figure 4). Thus, although the truncation of $p140^{HER2}$ is less extensive than in $p100^{neu}$, the effect of *HER2/c-erbB2* overexpression on PLC_γ could be due solely to this molecular species. To examine this possibility, we employed tumorigenic NIH-3T3 fibroblasts transfected with *HER2/c-erbB2* (HER2 cell line, Hudziak *et al.*, 1987). These cells express slightly over 10^6 receptors per cell, but in contrast to CER50 cells no $p140^{HER2}$ was present in the HER2 cells (Hudziak *et al.*, 1987). Nevertheless, like in CER50 cells, and unlike the parental NIH-3T3 cells, we observed tyrosine phosphorylation of PLC_γ in HER2 cells (Figure 5D, left panel). In addition, $p185^{HER2}$ molecules of HER2 cells co-immunoprecipitated with PLC_γ molecules. In contrast with CER50 cells, where trace amounts of $p140^{HER2}$ co-

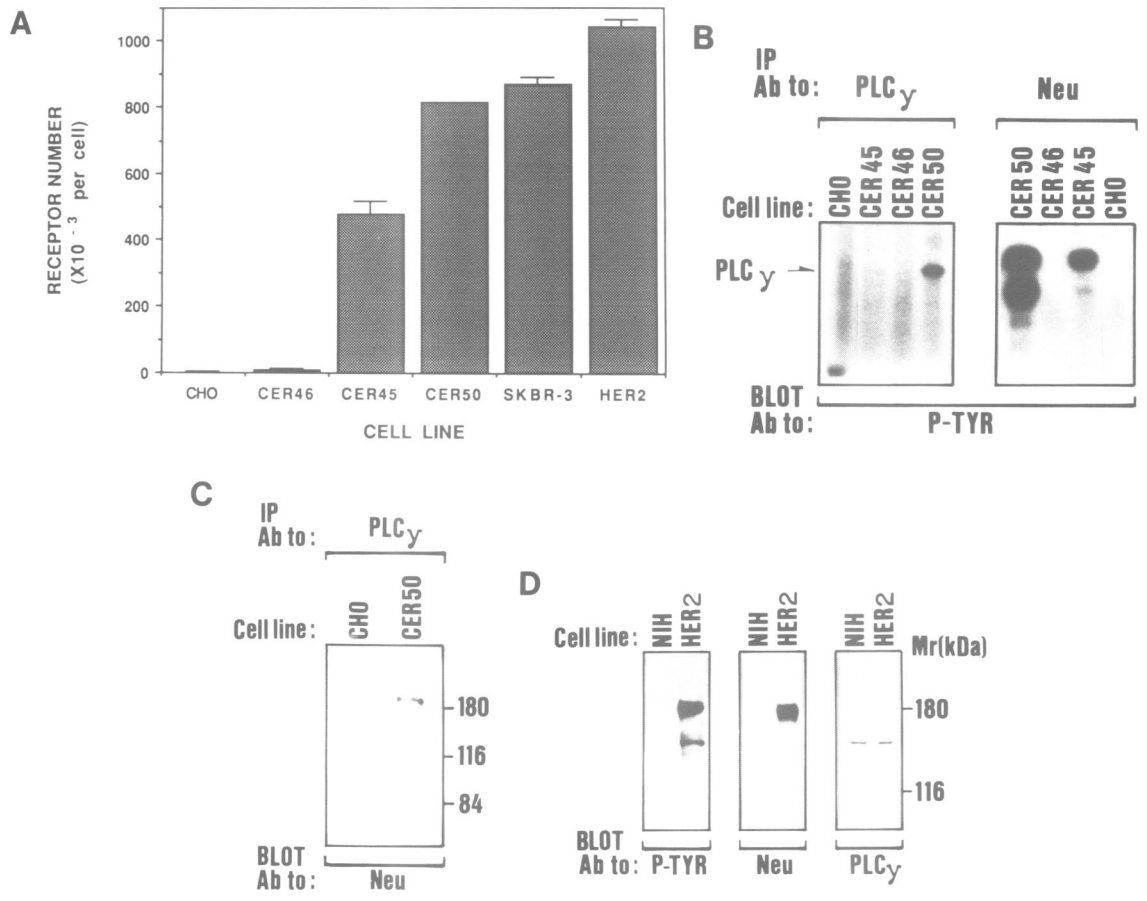

Fig. 5. PLC$_\gamma$ phosphorylation in *HER2/c-erbB2* overexpressing CHO and HER2 cells. (**A**) Level of expression of p185^{HER2} in transfectants. CHO cells were transfected with an expression vector that carries the human *HER2/c-erbB2* gene. Drug resistant clones were isolated and established as individual cell lines. 5×10^5 cells from each clone and also SKBR-3 human breast carcinoma cells and the HER2 fibroblasts (Hudziak *et al.*, 1987)/ were analysed by radioimmunoassay using a monoclonal antibody to HER2 protein (N28-IgG) as detailed under Materials and methods. (**B**) Tyrosine phosphorylation of p185^{HER2} and PLC$_\gamma$ in transfected clones. Cell lysates were prepared from 2×10^7 cells of the CHO sublines that express different levels of p185^{neu}. PLC$_\gamma$ and p185^{HER2} were immunoprecipitated with the corresponding antibodies PC4 and NCT, gel electrophoresed, and then transferred to nitrocellulose filters that were blotted with an antiserum to phosphotyrosine. Autoradiograms of [^{125}I]protein A labelled blots are shown. (**C**). Co-immunoprecipitation of p185^{HER2} with PLC$_\gamma$. Immunoprecipitates of PLC$_\gamma$ from either CHO or CER50 cells ($\sim 3 \times 10^7$ cells) were analysed by immunoblotting with an antibody to p185^{HER2} and detection with horseradish peroxidase conjugated protein A. (**D**) Analysis of HER2 cells. NIH-3T3 fibroblasts overexpressing the HER2−erbB-2 protein (2×10^7 cells) or control untransfected cells were pre-treated with vanadate and cell lysates prepared. These were subjected to immunoprecipitation with antibodies to PLC$_\gamma$ followed by Western blot analysis with antibodies to either phosphotyrosine or p185^{neu} (45% of the original lysate for each blot) or antibodies to PLC$_\gamma$ (5% of the lysate). Molecular masses of marker proteins are indicated.

immunoprecipitated with PLC$_\gamma$ (Figure 5C), HER2 cells exhibited co-precipitation of p185^{HER2} alone (Figure 5D). These observations support the possibility that overexpression of *HER2/c-erbB2*, rather than the appearance of p140^{HER2} is the cause of the effect on PLC$_\gamma$. In conclusion, high overexpression of p185^{HER2}, but not moderate expression levels, is correlated with constitutive tyrosine phosphorylation of both the putative receptor and PLC$_\gamma$. Based on comparison between the responses observed in CER50 and CER45 cells, we assume that a threshold above 5×10^5 receptors per CHO cell is required for the PLC$_\gamma$ effect to be seen.

Ligand-dependent phosphorylation and physical association of PLC$_\gamma$ with a chimeric neu receptor

The constitutive interaction of transforming neu−HER2 proteins with PLC$_\gamma$ suggested that ligand stimulation of the non-transforming protein will result in regulated association and phosphorylation of the effector enzyme. To examine this possibility, in the absence of a well-characterized neu ligand, we constructed a neu−EGF receptor chimera (denoted by NEC). In this protein, the extracellular ligand binding domain of the EGF receptor was linked to the transmembrane and the cytoplasmic portions of the rat neu protein. It has been previously shown that similar chimeras mediate neu-specific cellular effects (Pandiella *et al.*, 1989; Sistonen *et al.*, 1989), and lead to cellular transformation when ligand-occupied (Lee *et al.*, 1989; Lehvaslaiho *et al.*, 1989). An EGF receptor negative cell line (3T3 fibroblast line 2.2) was transfected with the NEC DNA constructs and a cell line that overexpresses the chimeric receptor (1.5×10^5 receptors per cell as determined by binding of radiolabelled EGF, data not shown) was selected. Preincubation of the NEC cells with vanadate followed by a 10 min exposure to a saturating concentration of EGF resulted in an increase in tyrosine phosphorylation of the p190^{nec} protein (Figure 6A, right panel). This was accompanied by an increased tyrosine phosphorylation of PLC$_\gamma$, which could be co-immunoprecipitated with a 190 kd band which was heavily phosphorylated on tyrosine residues (Figure 6A, left panel).

The latter protein was identified as $p190^{nec}$ by Western blotting with anti-$p185^{neu}$ antibodies (Figure 6A). This analysis also detected low association between PLC_γ and the chimeric receptor even in the absence of the stimulating ligand. As expected, the control parental 2.2 cells exhibited none of these effects in response to EGF. To examine the necessity of the pre-incubation with vanadate, we immunoprecipitated PLC_γ from EGF-stimulated NEC cells which had not been preincubated with the phosphatase inhibitor. As is evident from Figure 6B, tyrosine phosphorylation of PLC_γ and its physical association with $p190^{nec}$ are clearly detectable even in the absence of vanadate.

The specificity of the observed co-immunoprecipitation of PLC_γ with $p190^{nec}$ was tested by performing the immunoprecipitation, with anti-PLC_γ antibodies, in the presence of the 19-mer immunizing peptide used to raise the antibodies. The latter could partially abolish the association of PLC_γ with $p190^{nec}$ (Figure 6C), indicating that the $p190^{nec}$ signal in the Western blots is due to specific interaction with the enzyme.

The ligand-dependency of the PLC_γ effects was then examined by exposing NEC cells to increasing concentrations of EGF (Figure 7A). Although EGF at 5 ng/ml activated the receptor's tyrosine kinase, an effect on PLC_γ phosphorylation was not seen at EGF concentrations <50 ng/ml (Figure 7A). Nevertheless, increased association with PLC_γ, over the detectable basal association, was already seen with the lower concentrations of the ligand. Examination of the kinetics of interaction between the chimeric neu protein and PLC_γ revealed that phosphorylation of the receptor and PLC_γ and their physical association were already near maximal one minute after ligand stimulation (Figure 7B). At longer time intervals, the amount of tyrosine phosphorylated PLC_γ decreased despite the presence of vanadate in the medium (Figure 7B). In separate blotting experiments we confirmed that this decrease was due to reduced tyrosine phosphorylation, rather than to degradation of PLC_γ, thus suggesting the existence of vanadate-insensitive dephosphorylation. Nevertheless, the amount of $p190^{nec}$ that co-immunoprecipitated with PLC_γ remained unchanged ($\sim 1\%$ of $p190^{nec}$ molecules). Quantitative analysis revealed that 3 min after stimulation of NEC cells, 25% of the cellular PLC_γ molecules were phosphorylated on tyrosine residues (Figure 7C). For comparison, the extent of PLC_γ phosphorylation mediated by the wild-type EGF-receptor in A431 cells was estimated to be 35% of the total PLC_γ molecules in the cell (Figure 7C).

Discussion

This study addressed the possibility that diverse genetic mechanisms of oncogenic activation of the *neu–HER2* proto-oncogene converge at a common effector biochemical pathway. The genetic mechanisms which are involved in releasing the transforming potential include a specific point mutation (Bargmann *et al.*, 1986), extensive deletions of non-catalytic portions (Di Fiore *et al.*, 1987; Bargmann and Weinberg, 1988a), overexpression of the apparently normal gene (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987; Di Marco *et al.*, 1990) and ligand stimulation in the context of a chimeric receptor (Lee *et al.*, 1989; Lehvaslaiho *et al.*, 1989). Our present results indicate that in cultured cells that

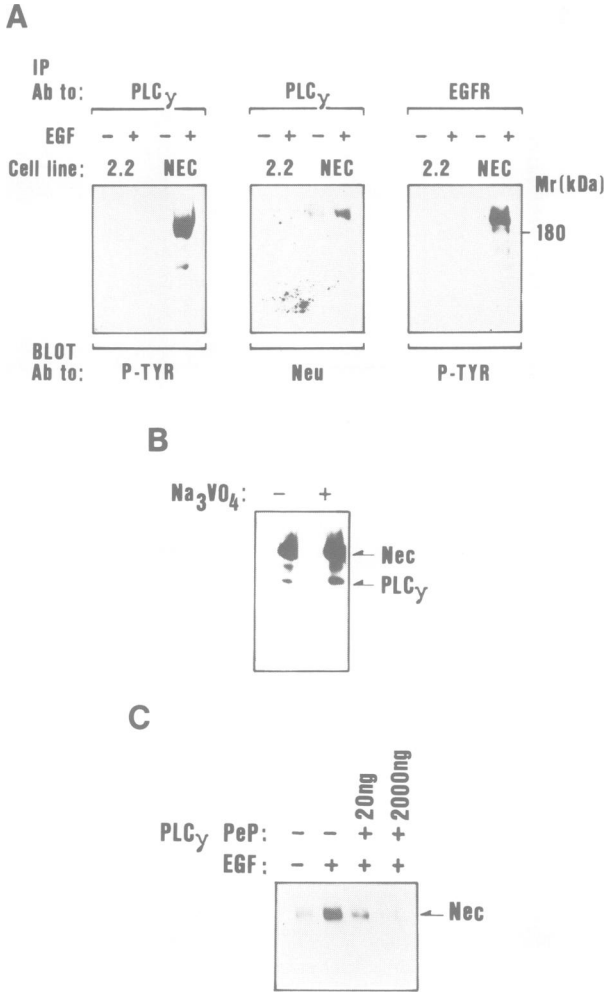

Fig. 6. Demonstration of ligand-dependent interaction of the neu–HER2 tyrosine kinase with PLC_γ. **(A)** The cell lines analysed were control untransfected 3T3 fibroblasts that do not express endogenous EGF receptor (2.2 cells), and NEC cells that express the EGF receptor–neu chimeric protein. Confluent monolayers of each cell line growing in 15 cm dishes were preincubated with vanadate (50 μM, for 2 h at 37°C) and then treated with or without EGF (100 ng/ml) for 5 min at 37°C. Cell lysates were then prepared and subjected to immunoprecipitation with either antibodies to PLC_γ or to EGF receptor (528-IgG, Kawamoto *et al.*, 1983). Following gel electrophoresis and transfer to nitrocellulose filters, the filters were blotted with antibodies to phosphotyrosine (PT5 antiserum) or an antibody to $p185^{neu}$ (NCT antiserum). The blots were detected as described in the legend to Figure 1. Autoradiograms (5 min exposure) of the chemiluminescence-generated signals are shown and the locations of standard mol. wt proteins are indicated in kilodaltons. **(B)** Vanadate effect on ligand-induced PLC_γ phosphorylation. NEC cells were treated with or without 50 μM Na_3VO_4 (2 h, 37°C) as indicated and then stimulated with 100 ng/ml EGF for 5 min at 37°C. PLC_γ was immunoprecipitated from cell lysates. Immunocomplexes were separated by SDS gel electrophoresis and transferred to nitrocellulose. Immunoblotting with anti-phosphotyrosine antibodies was performed as described in part A. The locations of $p190^{nec}$ and PLC_γ are indicated. **(C)** Inhibition of the co-immunoprecipitation of PLC_γ and the NEC chimeric receptor by a synthetic peptide. NEC cells were treated with or without EGF (100 ng/ml) for 5 min at 37°C as indicated. Then cell lysates were prepared and subjected to immunoprecipitation with antibodies to PLC_γ in the presence of the indicated concentrations (in ng/ml) of the 19 amino acid peptide that was originally used to generate the PLC_γ antiserum. Washed immunocomplexes were separated by gel electrophoresis followed by immunoblotting with anti-neu antibodies. Blots were detected with horseradish peroxidase-conjugated protein A (see Materials and methods).

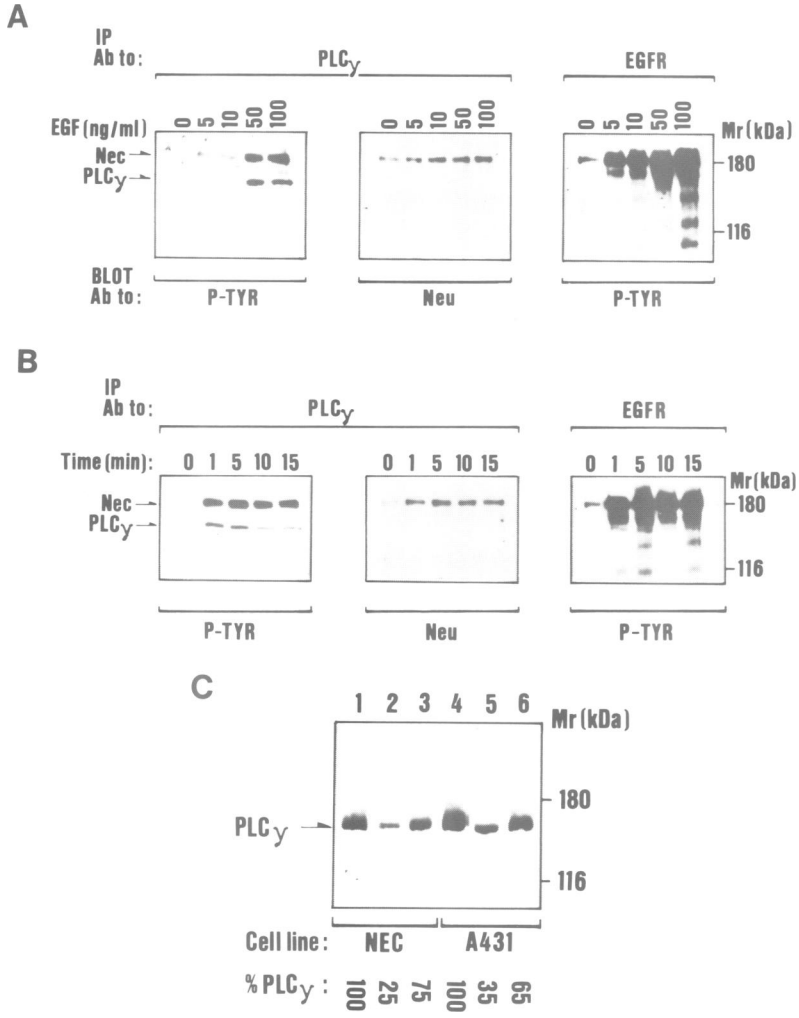

Fig. 7. (**A**) Ligand concentration-dependency of PLC$_\gamma$ interaction with the p190^{nec} tyrosine kinase. Confluent monolayers of NEC cells that express the EGF receptor–neu chimera were treated for 5 min at 37°C with the indicated concentrations of EGF. Cell lysates (1.5×10^7 cells) were subjected to immunoprecipitation with the antibodies indicated above the panels. Following gel electrophoresis and transfer to nitrocellulose filters, immunoblotting was performed with the antibodies indicated below each panel. (**B**) Kinetics of PLC$_\gamma$ interaction with the NEC chimeric receptor. Confluent monolayers of NEC cells in 15 cm dishes were treated at 37°C with 100 ng/ml EGF for the indicated periods of time. PLC$_\gamma$ and the NEC protein were then analysed by immunoprecipitation with the antibodies indicated above each panel, followed by Western blotting with the antibodies indicated below the panels. Chemiluminescence-based detection of the blots was performed as described in Materials and methods. (**C**) Relative proportions of tyrosine-phosphorylated PLC$_\gamma$ in stimulated NEC and A431 cells. 3×10^7 cells of each cell line were treated at 37°C with EGF (100 ng/ml) for 3 min, and cell lysates prepared. Half of each lysate was subjected to immunoprecipitation with antibodies to PLC$_\gamma$ (lanes 1 and 4) and the other half immunoprecipitated with agarose-immobilized antibodies to phosphotyrosine (lanes 2 and 5). The supernatants remaining after removal of the phosphotyrosine containing proteins were then reacted with antibodies to PLC$_\gamma$ (lanes 3 and 6). All the washed immunocomplexes were resolved by gel electrophoresis which was followed by Western blot analysis with antibodies to PLC$_\gamma$. The blot was detected with horseradish peroxidase-conjugated protein A. The percentages of PLC$_\gamma$ in each lane were determined by densitometry and are given at the bottom of the panel.

express either one of the four transforming neu–HER2 proteins, phosphoinositidase C$_\gamma$ molecules undergo increased tyrosine phosphorylation. In all these cases the transforming neu–HER2 proteins themselves displayed constitutively high phosphorylation on tyrosine residues, and underwent co-immunoprecipitation with PLC$_\gamma$, implying physical associations between these two molecules. A simple interpretation of these observations is that the different transforming forms of the neu–HER2 protein are functionally equivalent to a ligand-stimulated receptor which undergoes coupling to PLC$_\gamma$ following activation of its intrinsic tyrosine kinase. As tyrosine-phosphorylated PLC$_\gamma$ mediates accelerated turnover of phosphoinositides (Margolis *et al.*, 1990b; Nishibe *et al.*, 1990), this model predicts that in the various *neu*-transformed cells, protein kinase C and Ca^{2+} fluxes would be constitutively activated. This may in turn result in accelerated cellular proliferation, which is characteristic to the transformed phenotype (reviewed by Whitman and Cantley, 1988; Berridge and Irvine, 1989; Nishizuka, 1989). Certainly causative relationships between PLC$_\gamma$ modification and neu–HER2 mediated cellular transformation should await studies with specific inhibitors of PLC$_\gamma$, or employment of receptor mutants that do not couple to PLC$_\gamma$.

Quantitative analyses of the interaction between p185^{neu} and PLC$_\gamma$ revealed the following picture: ~25% of the cellular PLC$_\gamma$ molecules become phosphorylated on tyrosine residues after short stimulation of the *neu*-encoded tyrosine kinase (Figure 7C). Under the same conditions, the EGF receptor causes modification of 33% of PLC$_\gamma$

molecules in A431 cells (Figure 7C, and Wahl *et al.*, 1990). For comparison, the extent of modification of PLC_γ after stimulation of the PDGF receptor can reach 50–70% (Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989b). On the other hand, only 1% of the cellular PLC_γ molecules are found in association with the active $p185^{neu}$ kinase (Figure 1C). A similar degree of association between PLC_γ and the EGF receptor was reported in EGF-stimulated cells (Margolis *et al.*, 1989). We estimate that up to 2% of $p185^{neu}$ molecules co-immunoprecipitate with PLC_γ (data not shown). Again, this figure is similar to the one reported for the EGF receptor (Margolis *et al.*, 1989), but it is smaller than the reported number for the PDGF receptor (5% of the receptors found in association with PLC_γ; Morrison *et al.*, 1990).

An important open issue is whether the *neu–HER2*-encoded receptor directly phosphorylates PLC_γ. The following observations suggest that this is indeed the case: (i) both molecules strongly associate with each other following kinase activation; (ii) PLC_γ phosphorylation is very rapid (occurring within <1 min); and (iii) cells expressing a kinase defective mutant of $p185^{neu}$ do not display PLC_γ phosphorylation on tyrosine residues. By analogy, the observations that the neu-related receptors for EGF and PDGF phosphorylate PLC_γ *in vitro* (Nishibe *et al.*, 1989; Meisenhelder *et al.*, 1989; Kim *et al.*, 1990) also support direct p185-PLC_γ phosphorylation.

Although physical association between PLC_γ and the neu–HER2 tyrosine kinase was strongly dependent on catalytic activation of the latter enzyme, limited association was apparent even with the unstimulated kinase. This was seen in NE19 cells that express the normal rat *neu* gene (Figure 1B, right panel), and in cells that express the unstimulated NEC chimera (Figures 6 and 7). This limited association could be due to the low basal receptor autophosphorylation, as no association was found in cells that express a kinase-defective mutant of the receptor (Figure 3). PLC_γ was found to be associated also with the unstimulated EGF receptor (Margolis *et al.*, 1989) but not with the unstimulated PDGF receptor (Meisenhelder *et al.*, 1989), probably reflecting functional similarity between the structurally related EGF receptor and $p185^{neu}$. On the other hand, tyrosine phosphorylation of the other complex partner, namely PLC_γ, is probably not essential for physical association with the tyrosine kinase. This conclusion was inferred from experiments with the chimeric NEC protein: firstly, at low EGF concentrations PLC_γ was not detectably tyrosine phosphorylated but it formed a complex with the receptor (Figure 7A). Secondly, after a relatively long exposure to the ligand, significant dephosphorylation of PLC_γ took place with no decrease in the extent of association with the chimeric receptor (Figure 7B). It is relevant that the form of PLC_γ associated with the EGF receptor was found to be primarily the non-tyrosine-phosphorylated enzyme (Margolis *et al.*, 1990a). Whether this form, in the case of $p185^{neu}$, represents an initial intermediate of the complexed PLC_γ or a dephosphorylation product is currently unknown.

By analogy to other receptor tyrosine kinases (Ullrich and Schlessinger, 1990), it is conceivable that PLC_γ is but one of several cytoplasmic enzymes which stably associate with the activated tyrosine kinase of neu–HER2. Identification of the components of this complex may be instrumental for understanding the mechanism of cellular transformation by the *neu–HER2* oncogene.

Materials and methods

Cell culture

The following previously described cell lines were used: B104-1-1 cells (Bargmann *et al.*, 1986), HER2 (Hudziak *et al.*, 1987), and SKBR-3 human breast carcinoma cells (from the American Type Culture Collection). All cell lines were grown in Dulbecco's Modified Eagle (DME) medium containing 10% fetal calf serum. For immunoblotting experiments, monolayers were grown on fibronectin coated dishes (10 μg/ml fibronectin in phosphate buffered saline, PBS). Once cells were confluent, the medium was replaced with fresh medium containing 0.1% dialyzed fetal calf serum (dialysis was against PBS, membrane cut-off: 10–15 kd). Ten to twelve hours later, sodium orthovanadate (0.05 mM) was added and the cells were incubated for 1–2 h before experimentation. For metabolic labelling confluent monolayers of cells were incubated for 16 h at 37°C with 50 μCi/ml [^{35}S]methionine (NEN) in methionine-free medium supplemented with 1% dialysed fetal calf serum.

Construction of the NEC expression vector and establishment of NEC expressing cells

To construct an EGF receptor–*neu* chimeric DNA, we used the previously described EGF receptor–*kit* chimeric DNA (Lev *et al.*, 1990) which encodes the whole extracellular domain of the EGF receptor fused to the human *kit* DNA. This DNA was cloned into pBluescript plasmid with its 3′ end flanking the *Kpn*I site of the plasmid. A 600 bp *Bst*EII–*Kpn*I DNA fragment which encodes most of the cytoplasmic portion of $p145^{kit}$ was removed and replaced with the following double-stranded synthetic oligonucleotide:

5′-GTCACCAGCCGGCCACATATGTCGGTAC-3′
3′-GTCGGCCGGTGTATACAGC-5′

Proper insertion of the oligonucleotide was verified by nucleotide sequence analysis. This oligonucleotide contained an internal *Nae*I site which was used to delete further a 1.5 kb *Nae*I DNA fragment which contained the rest of the *kit* DNA. The resulting intermediate plasmid, SK-EC, contained an *Xho*I–*Nae*I portion of the EGF receptor fused in-frame to codons derived from the synthetic oligonucleotides designed to create a fusion segment with the unique *Nde*I site at the membrane–extracellular junction of the rat neu protein (Bargmann *et al.*, 1986). A 2.0 kb *Nde*I–*Kpn*I DNA fragment coding for the transmembrane and cytoplasmic portions of $p185^{neu}$ was cut out of a pBluescript vector and used to replace, by ligation, a heptanucleotide *Nde*I–*Kpn*I segment of the original synthetic oligonucleotide in the SK-EC plasmid, to yield SK-NEC. The fusion point was sequenced to ensure integrity of the open reading frame. The resulting chimeric DNA was cut out of SK-NEC using *Sal*I and cloned into the compatible *Xho*I site of a mammalian expression vector downstream of a cytomegalovirus promoter to yield the pCMV-NEC expression vector.

NIH-3T3 cells (2.2 subline), which lack endogenous EGF receptor, were transfected by the calcium phosphate precipitation method (Graham and Van der Eb, 1973) with both pCMV-NEC and a plasmid carrying the neomycin resistance gene. Following transfection a glycerol shock (15% glycerol in PBS for 3 min) was given after 4 h and the cells were subcultured into medium containing 0.5 mg/ml G418 (Gibco, Bethesda, MD). Resistant colonies were individually grown and assayed for binding of radiolabelled EGF. The NEC cell line, which expresses 1.5×10^5 receptors per cell, was selected for experimentation.

Establishment of HER2-expressing cell lines

Chinese hamster ovary (CHO) cells deficient in the DHFR gene (Urlaub and Chasin, 1980) were co-transfected with a plasmid that contained the HER2 gene under the control of the SV40 promoter (Hudziak *et al.*, 1987), and the pN1012 plasmid harbouring DHFR and the neomycin resistance genes. The calcium phosphate precipitation method of DNA transfer was used. After transfection, cells were grown in nucleotide-free MEM-α medium containing 0.5 mg/ml G418 (Gibco), 10% dialysed calf serum, 200 μg/ml proline and antibiotics. Resistant colonies were isolated, and separately cultured in medium containing increasing concentrations of methotrexate at the range of 0.1–3 μM. The expression of the HER2 protein in individual clones was determined by radioimmunoassay of living cells using a monoclonal antibody to HER2 (Stancovski,I., Hurwitz,E., Leitner,O., Ullrich,A., Yarden,Y. and Sela,M., submitted) as has been previously

described (Yarden, 1990). Receptor levels were expressed as numbers of receptors per cell using SKBR-3 cells as reference (Kraus *et al.*, 1987).

Construction of a kinase-defective mutant

The ATP binding site was predicted at Lys758 of the *neu* gene (Weiner *et al.*, 1989). To mutate this codon, we cloned a *StuI* fragment (nucleotide numbers 1368–3780 according to Bargmann *et al.*, 1986) of the transforming *neu* cDNA into a pBluescript plasmid (SK$^+$, Stratagene) at the *SmaI* site of the plasmid. The AAG codon of lysine, included in the cloned fragment, was mutated to an alanine codon (GCG) by using the synthetic oligonucleotide 5'-CCGTGGCTATCGCGGTGTTGAGAG-3' (the alanine codon is underlined) and site-directed mutagenesis on single-stranded DNA. The mutation was verified by nucleotide sequence analysis. The plasmid bearing the mutation was then cleaved with *NdeI* and *NcoI* to obtain a fragment corresponding to nucleotides 1895–3027 of the full-length rat *neu* cDNA and containing the mutation. This piece of DNA was inserted into an intermediate pBluescript vector containing the rest of the *neu* cDNA. Finally, a *SalI* fragment of 3.9 kb was removed from the latter plasmid and inserted into the *XhoI* site of a mammalian expression vector (pLSV/DHFR, Lev *et al.*, 1990) downstream of an SV40 promoter. The mutated (Ala758) and wild-type cDNAs, both in the pLSV vector, were co-transfected into NIH-3T3 fibroblasts (2.2 subline). Drug selection was used to obtain the cell lines RB22 (Lys758) and K758A (Ala758).

Antibodies

The NCT antiserum was raised against a synthetic peptide comprising the 15 carboxy-terminal amino acids of the human neu–HER2 protein. Antisera PC_2 and PC_4 against PLC$_\gamma$ were generated in rabbits injected with a 19 amino acid synthetic peptide corresponding to residues 1257–1275 of PLC$_\gamma$ (Stahl *et al.*, 1988). Peptides were chemically cross-linked to keyhole limpet haemocyanin (KLH, Calbiochem) using 1-ethyl-3 (3-diaminopropyl) carbodimide (EDAC, Pierce), and injected at 3 week intervals into 3 month-old rabbits. Antiserum to phosphotyrosine was raised in rabbits injected with KLH coupled either to phosphotyrosine alone or to polymerized phosphotyrosine-alanine-glycine as described (Kamps and Sefton, 1988). Affinity-purified antibodies were obtained by adsorption to immobilized phosphotyrosine.

Immunoprecipitation and immunoblotting analyses

Cell lysates were prepared after a brief wash with PBS and solubilization in SBN lysis buffer: 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP40, 1 mM ethylene glycol-bis (β-aminoethylether)-*N,N,N'-N'*-tetraacetic acid (EGTA), 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM sodium orthovanadate and 50 mM NaF. Cell lysates were cleared at 4°C by centrifugation (12 000 *g* × 10 min) and antibodies coupled to Sepharose–protein A beads (Pharmacia) were added. Immunocomplexes were precipitated after 1–2 h at 4°C and washed twice with the TNL' solution (50 mM Tris–HCl pH 7.5, 1% NP40, 0.5 M $LiCl_2$, 0.1 mM sodium orthovanadate and 50 mM NaF), once with TNL' solution without detergent and once with 50 mM Tris–HCl pH 7.5, 0.1 mM sodium orthovanadate. Washed immunoprecipitates were mixed with sodium dodecyl sulphate (SDS) gel sample buffer, heated at 95°C for 5 min and subjected to electrophoresis on 7.5% acrylamide gels. After electrophoresis, the gel-separated proteins were electrophoretically transferred onto nitrocellulose filters. Filters were first saturated for 1 h at 22°C with modified 'blotto' solution (10% dry low fat milk, 5% FCS, 1% ovalbumin in 20 mM Tris–HCl pH 7.6 and 137 mM NaCl). Antisera were then added in the same solution and incubation was continued for 1 h. For detection the filters were washed extensively with TTBS solution (0.05% Tween-20 in 20 mM Tris–HCl pH 7.6 and 17 mM NaCl) and reacted for 45 min at 22°C with either [^{125}I]protein A (Figure 5B) or with horseradish peroxidase-conjugated protein A. In the latter case the enzyme was removed by four washes with TTBS, the filters were reacted for 1 min with chemiluminescence reagent (ECL, Amersham) and exposed to autoradiography film (Hyperfilm-MP, Amersham) for 0.5–30 min. Immunoprecipitation of metabolically labelled proteins and *in vitro* kinase assays were performed as previously described (Goldman *et al.*, 1990). To enable quantitative comparison, lysates containing equal amounts of acid-insoluble radioactivity were used. All other immunoprecipitation experiments were performed after determination of the total protein concentration in the cell lysates, using a Biorad protein assay kit.

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