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Regulated Coupling of the *Neu* Receptor to Phosphatidylinositol 3′-Kinase and Its Release by Oncogenic Activation*

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The *neu* protooncogene encodes a tyrosine kinase receptor that is involved in the regulation of normal growth and malignant transformation. To circumvent the use of the incompletely characterized ligand of *Neu*, we constructed a chimeric protein composed of the ligand-binding domain of the epidermal growth factor receptor and the transmembrane and cytoplasmic portions of *Neu*. By expressing this *Neu*-epidermal growth factor receptor chimera (termed NEC), we found that following stimulation by the heterologous ligand, the tyrosine kinase of *Neu* became associated with a phosphatidylinositol (PI) kinase activity. The association was dependent on the concentration of the ligand and was almost maximal within 30 s after ligand binding. The lipid kinase was identified as a type I PI 3′-kinase on the basis of its inhibition by Nonidet P-40 and high pressure liquid chromatography of the phosphorylated product. To confirm the identification of PI 3′-kinase as an effector of *Neu*, we raised antibodies to the α-isoform of the regulatory subunit of PI 3′-kinase (p85). Using these antibodies, it was possible to directly demonstrate ligand-dependent formation of a tyrosine-phosphorylated complex of NEC and PI 3′-kinase. Apparently, both PI 3′-kinase and phospholipase C_γ, another substrate of the *Neu* kinase, simultaneously associated with the same activated NEC molecule. Nevertheless, immunofluorescence localization of PI 3′-kinase revealed no significant cellular redistribution of the enzyme after activation of the *Neu* kinase. Interestingly, PI 3′-kinase was localized primarily to the cell nucleus and to confined regions of the plasma membrane. Analysis of mutants of the *Neu* protein indicated that the oncogenic point-mutated *Neu* (Glu^{664}) was permanently coupled to PI 3′-kinase; but two nontransforming versions of the oncoprotein, a kinase-defective protein and a carboxyl-terminally deleted *Neu*, were devoid of the constitutive association with PI 3′-kinase. Hence, we concluded that phosphatidylinositol 3′-kinase is a physiological substrate of the *Neu* receptor, but the regulation of this coupling is released upon oncogenic activation.

The *neu* gene (also called erbB-2 and HER-2) encodes a 185-kDa transmembrane glycoprotein that is structurally related to receptors for polypeptide growth factors and particularly to the receptor for the epidermal growth factor (EGF)[1] (reviewed by Yarden and Ullrich (1988)). The similarity is greatest in the intracellular domains of these two proteins, where they share an intrinsic tyrosine kinase activity. Despite their homology, distinct mitogenic signals appear to be generated by each receptor (Di Fiore *et al.*, 1990). In contrast to the EGF receptor, overexpression of *neu* in murine fibroblasts confers a transformed phenotype (Hudziak *et al.*, 1987; Di Fiore *et al.*, 1987). The strong oncogenic potential of *neu* is also reflected by a point mutant of the gene that was induced in rats treated with a chemical carcinogen (Bargmann *et al.*, 1986a). As a result of this mutation, the enzymatic activity of $p185^{neu}$ is dramatically elevated both *in vitro* (Bargmann and Weinberg, 1988) and in living cells (Yarden, 1990), probably as a result of constitutive receptor dimerization (Weiner *et al.*, 1989). These observations, together with the fact that the transforming $p185^{neu}$ undergoes accelerated endocytosis and degradation in the cell (Stern *et al.*, 1988; Yarden, 1990), raise the possibility that the mutation functionally mimics the effect of the endogenous ligand of *Neu*.

The first clue to the existence of a distinct ligand that binds to the *Neu* protein was obtained by using a series of bioassays and medium conditioned by *ras*-transformed fibroblasts (Yarden and Weinberg, 1989). This activity was partially purified and found to correspond to a 35-kDa heat-resistant glycoprotein (Yarden and Peles, 1991). An apparently similar molecule was isolated from medium conditioned by human breast cancer cells (Lupu *et al.*, 1990). Despite this progress in the isolation of the *Neu* ligand, the lack of sufficient quantities of a homogeneously pure molecule limited studies of signal transduction by $p185^{neu}$. Detailed analysis of this mechanism is thought to provide better understanding of the role played by $p185^{neu}$ in human adenocarcinomas that frequently overexpress this receptor (reviewed by Slamon *et al.* (1989) and Gullick (1990)). Attempts to circumvent this problem have utilized agonistic monoclonal antibodies (Yarden, 1990; Scott *et al.*, 1991; Stancovski *et al.*, 1991) and chimeric receptors in which the ectodomain of $p185^{neu}$ was replaced by the corresponding region of the EGF receptor (Lee *et al.*, 1989; Lehvaslaiho *et al.*, 1989; Peles *et al.*, 1991; Fazioli *et al.*, 1991). This latter approach has been particularly useful as it revealed that $p185^{neu}$ has all the biochemical attributes of a growth factor receptor, including potent mitogenic activity and induction of elevated transcription of nuclear protooncogenes (Sistonen *et al.*, 1989). Moreover, $p185^{neu}$ shares with other growth factor receptors the ability to induce both a rapid

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[1] The abbreviations used are: EGF, epidermal growth factor; PI, phosphatidylinositol; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PIP, phosphatidylinositol phosphate; MBS, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide.

increase in the concentration of intracellular calcium and an elevated rate of hydrolysis of polyphosphoinositides (Pandiella *et al.*, 1989). These events may be due to a ligand-dependent coupling of p185^{neu} with a phosphoinositide-specific phospholipase (phospholipase C_γ) that physically associates with the activated tyrosine kinase (Peles *et al.*, 1991; Fazioli *et al.*, 1991). Another signaling molecule that undergoes tyrosine phosphorylation following ligand binding to a chimeric *Neu* protein is the GTPase-activating protein of *ras* (Fazioli *et al.*, 1991). In these respects, p185^{neu} appears to share with other receptors the formation of a "signaling complex" composed of several second messenger-producing proteins (see Kaplan *et al.*, 1990; reviewed by Yarden and Kelman (1991)).

A protein that almost invariably participates in the signaling of receptor tyrosine kinases is phosphatidylinositol (PI) 3'-kinase (reviewed by Cantley *et al.*, 1991). This enzyme catalyzes phosphorylation at D-3 of the inositol ring of phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate (Auger *et al.*, 1989) to generate PI metabolites with yet unknown physiological functions. The possibility that PI 3'-kinase is an effector of the *Neu* receptor was raised by the observation that the homologous receptor for EGF is coupled to this lipid kinase (Bjorge *et al.*, 1990). In addition, PI 3'-kinase is probably involved in cell growth regulation. A correlation exists between the ability of p60$^{v\text{-}src}$ and middle T mutants to induce transformation and their capacity to associate with PI kinase activity (Fukui and Hanafusa, 1989; Talmage *et al.*, 1989); and a mutant PDGF-β receptor (Coughlin *et al.*, 1989), but not a similar mutant of the PDGF-α receptor (Yu *et al.*, 1991) that lacked the ability to associate with PI 3'-kinase, failed to induce DNA synthesis in response to PDGF. These observations led us to test the possibility that phosphatidylinositol 3'-kinase is an effector protein that participates in signal transduction by the normal p185^{neu} and its oncogenic mutant. In this report, we addressed this question by using a chimeric *Neu* protein that underwent stimulation by a heterologous ligand. We also used the normal and the transforming p185^{neu} proteins and their mutants. Our results indicate that PI 3'-kinase is a physiological target of both ligand-stimulated and mutation-activated *Neu* tyrosine kinase.

EXPERIMENTAL PROCEDURES

Materials

EGF was supplied by Biomakor (Rehovot, Israel) and by Toyobo (New York). [γ-^{32}P]ATP (3000 Ci/mol) and [^{3}H]glycerophosphoinositol 4-phosphate were from Amersham Corp. Protein A-Sepharose was from Pharmacia LKB Biotechnology Inc. or was prepared in our laboratory. Thin-layer chromatography plates were from Merck. The Partisphere SAX anion-exchange column was from Whatman. Fetal bovine serum and calf serum were the products of HyClone Laboratories (Logan, Utah). The ECL chemiluminescence kit used for immunoblotting was purchased from Amersham Corp. Molecular weight standards for gel electrophoresis were from Bio-Rad. All other chemicals were purchased from Sigma.

Buffered Solutions

HNTG buffer contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% (w/v) Triton X-100, and 10% glycerol. Solubilization buffer contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% (w/v) Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 50 mM NaF, 2 mM sodium orthovanadate, 1 mM EGTA, aprotinin (0.15 trypsin inhibitor unit/ml), 1 mM phenylmethylsulfonyl fluoride. PBS contained 137 mM NaCl, 2.7 mM KCl, 7.9 mM $NaHPO_4$, and 1 mM KH_2PO_4 (pH 7.2). High wash contained 50 mM HEPES (pH 7.5), 500 mM NaCl, 0.1% SDS, 0.2% (w/v) Triton X-100, 5 mM EGTA, 20 mM NaF, 5 mM EDTA, and 2 mM NaV. Medium wash contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% SDS, 0.2% (w/v) Triton X-100, 5 mM EGTA, 20 mM NaF, 5 mM EDTA, and 2 mM NaV. Low wash contained 10 mM Tris (pH 8.0), 0.1% (w/v) Triton X-100, 20 mM NaF, 5 mM EDTA, and 2 mM NaV.

Cell Culture

All cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and antibiotics. For assays, the cells were grown to confluency on fibronectin-coated dishes (Nunc), and the medium was replaced 14 h before the experiment with fresh Dulbecco's modified Eagle's medium that contained only 0.1% calf serum.

Antibodies

Antibodies to p85α of phosphatidylinositol 3'-kinase were raised against a synthetic peptide comprising the 17 carboxyl-terminal amino acids of the 85-kDa subunit of bovine type I PI 3'-kinase (α-isoform) (Otsu *et al.*, 1991). A single cysteine residue was added to the carboxyl terminus of the peptide. After synthesis, the peptide was prepared for injection in the following different ways.

Glutaraldehyde-polymerized Peptide—15 mg of peptide were mixed with glutaraldehyde (0.1% final concentration in 0.1 M sodium phosphate (pH 8)). The mixture was stirred for 48 hr at 22 °C in the dark and then dialyzed against PBS.

MBS-conjugated Peptide—7.2 mg of MBS in 0.2 ml of *N,N'*-dimethylformamide were added dropwise to 40 mg of keyhole limpet hemocyanin (Calbiochem) in PBS and stirred for 30 min at 22 °C. The conjugates were separated from excess MBS by chromatography over a Sephadex G-25 column in 0.1 M sodium phosphate (pH 6.0), and 20 mg of peptide were immediately mixed with the peak fraction. The mixture was stirred for 48 h at 22 °C and then dialyzed against PBS.

1-Ethyl-3-(3'-dimethylaminopropyl)carbodiimide-conjugated Peptide—20 mg of peptide in 2 ml of PBS were mixed with 10 mg of 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide, and the mixture was incubated for 5 min at 22 °C. Then 20 mg of keyhole limpet hemocyanin were added, and the mixture was stirred for 48 hr at 4 °C, followed by dialysis against PBS. A mixture containing equal peptide equivalents of the three preparations (1 mg each) together with 1 mg of free peptide was mixed with Freund's complete adjuvant at a 1:1 (v/v) ratio and was injected into 4-month-old female rabbits. Booster injections were given at 2–3-week intervals in incomplete adjuvant, and the animals were bled 10 days after each injection. Sera obtained after the third booster injection were used in this study. The other antibodies we used have been previously described (Peles *et al.*, 1991). The NCT antiserum was directed to the 15 carboxyl-terminal amino acids of p185^{neu} (Yarden, 1990), whereas the J9 antiserum was specific to the corresponding peptide of the PDGF-β receptor (Yarden *et al.*, 1986), the PC_4 antiserum to phospholipase C_γ was generated in rabbits injected with a 19-amino acid synthetic peptide corresponding to residues 1257–1275 of phospholipase C_γ (Stahl *et al.*, 1988), and the antibodies to phosphotyrosine were raised in rabbits as described (Kamps and Sefton, 1988). Affinity-purified antibodies to phospholipase C_γ were obtained by adsorption to the corresponding synthetic peptide immobilized on Sepharose CL-4B (Pharmacia), extensive washing, and elution at low pH. The monoclonal antibody 528-IgG to the human EGF receptor was a gift from John Mendelsohn (Matsui *et al.*, 1984).

Establishment of Cell Lines Expressing Mutants of neu Gene

NIH-3T3 mouse fibroblasts devoid of endogenous EGF receptor were transfected using the calcium phosphate precipitation method. A plasmid that encoded a *Neu* protein and a plasmid carrying the neomycin resistance gene were cotransfected, and stably expressing cells were selected for growth in medium containing 0.5 mg/ml G418 (GIBCO). The following cell lines were used: NEC cells, which express a chimeric EGF receptor (extracellular domain)-p185^{neu} (transmembrane and cytoplasmic domains) (Peles *et al.*, 1991); NE19 cells, which overexpress the cDNA of the normal *neu* gene (Val^{664}) (Bargmann *et al.*, 1986b); K758A cells, which overexpress a kinase-negative mutant of p185^{neu} (Glu^{664}) in which the lysine residue at the nucleotide-binding site is replaced by alanine (Peles *et al.*, 1991); and ΔCT cells, which express a mutant *Neu* protein (Glu^{664}) in which the 255 carboxyl-terminal amino acids are deleted. A detailed description of the construction of the ΔCT mutant gene will be described elsewhere.[2]

[2] R. Ben-Levy *et al.*, manuscript in preparation.

The B104-1-1 cell line, which overexpresses the transforming $p185^{neu}$, has been previously described (Bargmann *et al.*, 1986b).

Preparation of Cell Lysates and Immunoprecipitation

After stimulation of cell monolayers with EGF, they were washed twice with ice-cold PBS and scraped with a rubber policeman into solubilization buffer (1 ml for 10^7 cells). The lysates were transferred into 1.5-ml centrifuge tubes, vortexed vigorously, and left on ice for 10 min. Clearance from nuclei and unbroken cells was done by centrifugation at 4 °C (10 min, 12,000 × *g*). Antibodies coupled to 3 mg of protein A-Sepharose beads were added; and the mixture was incubated for 1–2 h, with shaking, at 4 °C. Immunocomplexes were washed twice with the high, medium, and low washes, mixed with SDS gel sample buffer, heated at 95 °C for 5 min, and subjected to electrophoresis on 7.5% acrylamide gel.

Western Blotting

Gel-separated proteins were electrophoretically transferred onto a sheet of nitrocellulose at 200 mA for 1 h. Nonspecific binding was blocked by incubating the nitrocellulose for 1 h at 22 °C in a blocking solution (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 10% milk, and 0.01% Tween 20). The antibody was then added to the blocking solution, and the incubation was continued for an additional hour. The nitrocellulose filter was then washed three times (10 min each) with the blocking solution and incubated with horseradish peroxidase-conjugated protein A. Following 45 min at 22 °C, the reagent was removed by four washes as described above, reacted for 1 min with chemiluminescence reagent, and exposed to x-ray film for 0.5–30 min.

In Vitro PI Kinase Assay

Cells were grown to confluence in six-well trays (Nunc) and starved for 14 h in medium containing 0.1% fetal bovine serum. After stimulation with EGF, the cells were solubilized, and their lysates were subjected to immunoprecipitation as described above. The immunoprecipitates were then washed twice with each of the following buffers: (*i*) solubilization buffer; (*ii*) 0.5 M LiCl in 100 mM Tris-HCl (pH 7.5); and (*iii*) 0.1 M Tris-HCl (pH 7.5) containing 0.1 M NaCl and 1 mM EDTA. PI kinase activity in the washed immunoprecipitates was assayed according to Whitman *et al.* (1985). Reaction products were separated by TLC in chloroform, methanol, 2 M ammonium hydroxide (9:7:2); visualized by autoradiography, and identified by comparison with a nonlabeled PI phosphate standard. For experiments including Nonidet P-40, the immunoprecipitates were washed with PBS containing 1% Nonidet P-40 and assayed in the presence of 0.5% Nonidet P-40.

Deacylation and HPLC Analysis

The ^{32}P-labeled PIP products were scraped from the TLC plates and treated with methylamine (along with a ^{3}H-labeled phosphatidylinositol 4-phosphate standard) to remove the fatty acyl side chain as described by Whitman *et al.* (1988). The deacylated products were separated by HPLC using a Partisphere SAX anion-exchange column (Whatman) as described by Auger *et al.* (1989). Samples were loaded in water and eluted with 1 M $(NH_4)_2HPO_4$ (pH 3.8) at 1 ml min^{-1}. A gradient from 0 to 1 M $(NH_4)_2HPO_4$ (pH 3.8) was developed in 130 min using dual pumps (pump A (H_2O)/pump B (1 M $(NH_4)_2HPO_4$ (pH 3.8))). Pump B was run at 0% for 10 min, then to 25% pump B with a duration of 60 min, and then to 100% pump B for 50 min. Eluted fractions were quantitated by liquid scintillation counting.

Immunofluorescence

Confluent monolayers of cells growing on fibronectin-coated glass coverslips were incubated for 14 h in Dulbecco's modified Eagle's medium containing 0.1% fetal bovine serum. After stimulation with EGF, the cells were gently washed with PBS and fixed for 30 min at 4 °C in 3% paraformaldehyde. After an additional rinse with PBS, the cells were incubated for 5 min in PBS containing 5% Triton X-100 and then washed twice (10 min each) with PBS. The antibody to p85α or the control antiserum was added at a 1:200 dilution and was incubated with the cells at 22 °C for 30 min. Following washing with PBS as before, rhodamine-labeled goat antibodies to rabbit immunoglobulins (a gift from Benjamin Geiger) were added and allowed to react with the cells for 30 min at 22 °C. This was followed by another cycle of washing with PBS. Immunofluorescence was observed with a Zeiss fluorescent microscope and photographed with Kodak X-Omat 400 film.

RESULTS

Ligand-dependent Association of Phosphatidylinositol Kinase Activity with Chimeric Neu Receptor—We have recently described the biochemical characteristics of a 35-kDa glycoprotein that fulfills the criteria for being the ligand of the *Neu* receptor (Yarden and Peles, 1991). However, due to the limited availability of the purified putative ligand, we established an alternative cellular system for the study of the mechanism of signal transduction by *Neu* (Peles *et al.*, 1991). Replacement of the presumed ligand-binding domain of $p185^{neu}$ with the corresponding portion of the homologous receptor for EGF resulted in a chimeric protein that bound EGF and underwent catalytic activation. Overexpression of the chimeric receptor (termed NEC for *Neu*-EGF receptor chimera) in NIH-3T3 fibroblasts that lacked detectable endogenous receptor for EGF (cell line 2.2) indicated that upon binding of the heterologous ligand, not only the chimeric receptor, but also a series of other cellular proteins underwent increased phosphorylation on tyrosine residues (Fig. 1, *upper*). An apparently similar set of proteins underwent elevated phosphorylation on tyrosine residues in cells that expressed an oncogenic mutant of $p185^{neu}$ (Glu^{664}, B104-1-1 cells) (Fig. 1, *lower*), but not the wild-type protein (Val^{664}, NE19 cells). It has been shown previously that both phospholipase C_γ and the *ras* GTPase-activating protein (Peles *et al.*, 1991; Fazioli *et al.*, 1991) are included in this set of *Neu*-specific substrates. To explore the possibility that a third common effector of many receptor tyrosine kinases, namely phosphatidylinositol 3′-kinase (Cantley *et al.*, 1991), was also shared by *Neu*, we

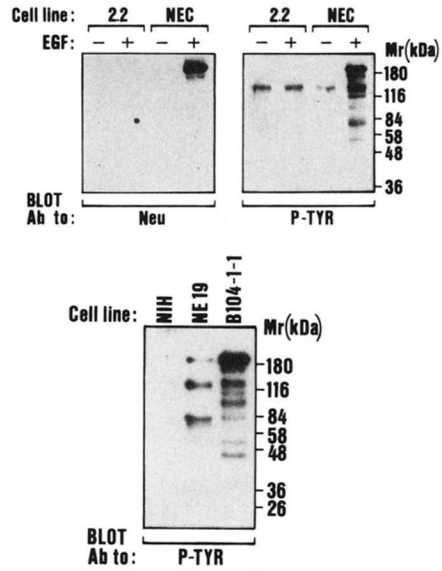

FIG. 1. **Tyrosine phosphorylation substrates of *Neu* kinase.** *Upper*, monolayers of 3T3 fibroblasts that express the chimeric *Neu* receptor (NEC cells) or their parental nontransfected cells (cell line 2.2) were treated or untreated with EGF (100 ng/ml) for 5 min at 37 °C. Cell lysates were then prepared and subjected to immunoprecipitation with antibodies to phosphotyrosine, followed by specific elution with phenyl phosphate. The eluants were resolved by gel electrophoresis and transferred onto nitrocellulose filters that were probed with either antibodies (*Ab*) to $p185^{neu}$ (*left*) or antibodies to phosphotyrosine (*P-Tyr*) (*right*). *Lower*, murine fibroblasts that overexpress the wild-type $p185^{neu}$ (NE19 cells), the Glu^{664} mutant (B104-1-1 cells), or control untransfected cells (NIH) were lysed and subjected to analysis of tyrosine phosphorylation as described (*upper*) by Western blotting. Detection of the blots was performed with peroxidase-conjugated protein A and a chemiluminescence reagent (ECL, Amersham Corp.). Shown are the resultant autoradiographs (3-min film exposure). The locations of molecular weight marker proteins are indicated.

used an *in vitro* phosphatidylinositol kinase assay (Whitman *et al.*, 1985) and applied it to immunoprecipitates of the NEC protein. The results of this experiment showed that phosphatidylinositol kinase activity was not associated with the unstimulated NEC protein. However, following a short exposure to the heterologous ligand, the enzymatic activity was found in immunocomplexes of the receptor that were obtained with antibodies to either the extracellular or the cytoplasmic domain (Fig. 2). Further characterization of the association between the ligand-activated *Neu* tyrosine kinase and the lipid kinase revealed that it was dependent on the concentration of the ligand, probably reflecting the gradual saturation of the binding sites (Fig. 3, *upper*). In analogy to other receptors for growth factors (Kaplan *et al.*, 1987; Coughlin *et al.*, 1989; Varticovski *et al.*, 1989; Bjorge *et al.*, 1990; Lev *et al.*, 1991), we observed a very rapid association between the two proteins; only 30 s after adding the ligand, an almost maximal PI kinase activity could be recovered in the immunoprecipitates (Fig. 3, *lower*). Important, the enzymatic activity decreased over longer periods of incubation with the ligand, even under conditions that maintained a high level of phosphorylation of the receptor on tyrosine residues (data not shown).

Identification of Phosphatidylinositol Kinase Activity as Type I Enzyme—The major phosphatidylinositol kinase in fibroblasts phosphorylates D-4 of the inositol ring (Whitman *et al.*, 1987). However, the lipid kinase that associates with activated tyrosine kinases is specific to position 3 of the sugar ring and was termed type I kinase (Whitman *et al.*, 1988). To examine the specificity of the NEC-associated PI kinase, we first tested its susceptibility to inhibition. As expected for a type I enzyme, the detergent Nonidet P-40 completely blocked the kinase, whereas adenosine (0.3 mM) had no inhibitory effect when added to the PI kinase reaction mixture (Fig. 4, *upper*). To further confirm the identification, we analyzed the PIP product by high pressure liquid chromatography on a strong anion-exchange resin (Varticovski *et al.*, 1989). Fig. 4 (*lower*) depicts the results of this analysis; the PIP product that was extracted from the thin-layer plate and deacylated

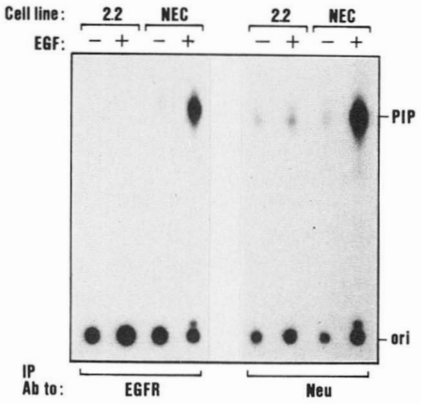

FIG. 2. **Ligand-dependent association of PI kinase activity with chimeric *Neu* receptor.** Monolayers of cells expressing the chimeric *Neu* receptor (NEC cells) or the parental untransfected cells (cell line 2.2) were incubated for 5 min at 37 °C with EGF (100 ng/ml). Cell lysates were then prepared and subjected to immunoprecipitation (*IP*) with either a monoclonal antibody (*Ab*) directed to the extracellular domain of the EGF receptor (*EGFR*) (528-IgG) or a rabbit antiserum to the carboxyl terminus of $p185^{neu}$ (NCT antibody). Following extensive washing of the immunoprecipitates, they were assayed for the presence of PI kinase activity by adding [γ-^{32}P]ATP and phosphatidylinositol. The reaction products were extracted and separated by thin-layer chromatography. An autoradiograph of the TLC plate is shown, and the locations of PIP and the origin (*ori*) are indicated.

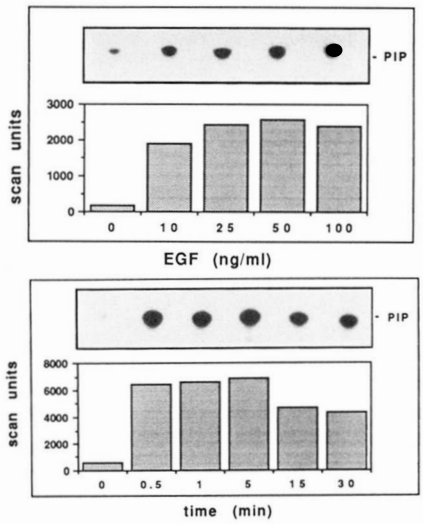

FIG. 3. **Ligand concentration and time dependencies of association of *Neu* with PI kinase activity.** NEC cells were treated at 37 °C with various concentrations of EGF for 5 min (*upper*) or with 100 ng/ml EGF for the indicated periods of time (*lower*). Cell lysates were prepared and subjected to immunoprecipitation with an antibody to the EGF receptor (528-IgG). PI kinase assay was performed on the washed immunoprecipitates, followed by TLC analysis of the reaction products. Shown are the portions of the TLC autoradiographs that correspond to the locations of PIP along with graphic illustrations of the relative levels of PIP as determined by densitometric scanning.

eluted earlier than a glycerophosphoinositol 4-phosphate standard, at a rate corresponding to the 3′-isomer. Taken together, the results shown in Fig. 4 identified the PI kinase that became associated with the NEC protein upon ligand binding as specific to D-3 of the inositol ring.

Coprecipitation of PI Kinase Activity with Phospholipase C_γ—To investigate the formation of a noncovalent complex between NEC and PI 3′-kinase, we examined the possibility that it was precipitable with antibodies specific to phosphotyrosine. Indeed, an increased enzymatic activity could be precipitated with anti-phosphotyrosine antibodies from cells that were pre-exposed to the ligand of NEC (Fig. 5, *upper*). The specificity of this immunoprecipitation was demonstrated by the ability of a phosphotyrosine analog, phenyl phosphate, at 10 mM to displace the enzymatic activity (data not shown), thus confirming that a tyrosine-phosphorylated complex of NEC and PI 3′-kinase was formed after ligand binding. We and others have previously shown that the activated *Neu* tyrosine kinase becomes associated with phospholipase C_γ that undergoes phosphorylation on tyrosine residues (Peles *et al.*, 1991; Fazioli *et al.*, 1991). This raised the possibility that phospholipase C_γ participated in the tyrosine-phosphorylated complex that included PI 3′-kinase and the receptor. This possibility was examined by assaying immunoprecipitates of phospholipase C_γ for the presence of PI kinase activity. The anti-phospholipase C_γ antibody that we used was specific to a synthetic peptide and recognized a single protein band of 145 kDa on Western blots (Peles *et al.*, 1991). This antiserum was affinity-purified over a column of the immunizing peptide, and the specific antibody was used for immunoprecipitation of phospholipase C_γ from lysates of ligand-stimulated NEC cells. As shown in Fig. 5 (*upper*), an elevated level of PI kinase activity was associated with the immunoprecipitate obtained from EGF-treated NEC cells. The specificity of this coimmunoprecipitation was further examined by using the preimmune rabbit serum or the immune serum in the presence of the immunizing peptide (Fig. 5, *lower*). In both cases, the

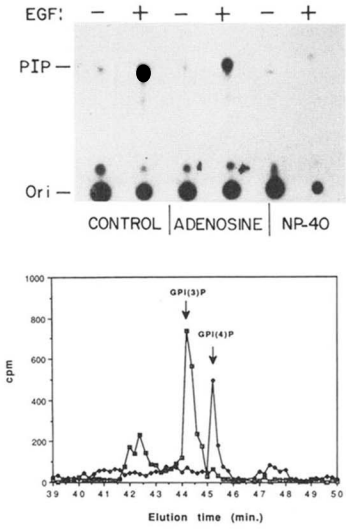

FIG. 4. **Identification of lipid kinase activity that associated with NEC receptor as type I PI kinase.** *Upper,* adenosine and Nonidet P-40 sensitivities of the lipid kinase. NEC cells were either untreated (−) or treated (+) with 50 ng/ml EGF for 1 min at 37 °C. Immunoprecipitates of the NEC receptor were then obtained and assayed *in vitro* for associated PI kinase activity. The assay mixture contained 0.3 mM adenosine or 0.5% (v/v) Nonidet P-40 (*NP-40*) as indicated. An autoradiograph of the TLC-separated lipid products is shown, and the locations of the origin (*Ori*) and PIP are indicated. *Lower,* HPLC analysis of the deacylated phosphatidylinositol phosphate. Serum-starved NEC cells were stimulated with 50 ng/ml EGF for 1 min at 37 °C. Following cell lysis and immunoprecipitation of the NEC protein, the immunocomplex was assayed for associated PI kinase activity, and the reaction products were separated by TLC as described (*upper*). The radioactive PIP spot was scraped from the plate, deacylated alongside a [^{3}H]phosphatidylinositol 4′-phosphate standard, and analyzed by ion-exchange chromatography. Fractions of 0.2 ml were collected, and their radioactivity was determined in both ^{3}H (♦) and ^{32}P (⊡) channels. The location of the marker deacylated glycerophosphoinositol 4′-phosphate (*GPI(4)P*) is shown together with the apparent location of glycerophosphoinositol 3′-phosphate (*GPI(3)P*).

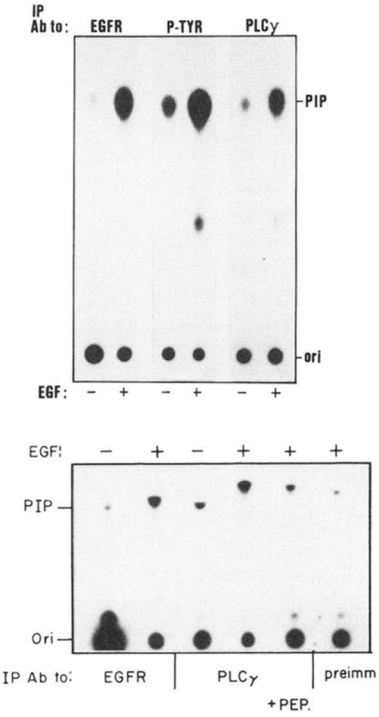

FIG. 5. **Immunoprecipitation of PI kinase activity with antibodies to phosphotyrosine and phospholipase C$_\gamma$.** *Upper,* monolayers of NEC cells grown on 90-mm plates were either stimulated for 5 min at 37 °C with EGF (100 ng/ml) or left untreated. Cell lysates were then prepared and subjected to immunoprecipitation (*IP*) with antibodies (*Ab*) to the EGF receptor (*EGFR*) (528-IgG), antibodies to phosphotyrosine (*P-TYR*), or affinity-purified antibodies to phospholipase C$_\gamma$ (*PLC$_\gamma$*). *Lower,* NEC cells were treated as described (*upper*), and the cell lysates were subjected to immunoprecipitation with antibodies to the EGF receptor, a rabbit antibody to phospholipase C$_\gamma$, or a preimmune serum obtained from the same animal (*preimm.*). As a control, the synthetic peptide that was used to immunize rabbits against phospholipase C$_\gamma$ was added at 0.1 mg/ml to the immunoprecipitation reaction (*+PEP*). All the immunoprecipitates were assayed for the presence of PI kinase activity by *in vitro* phosphorylation of PI, followed by TLC separation of the lipid products. The autoradiographs of the TLC plates are shown, and the locations of the origin (*ori*) and PIP are indicated.

immunocomplexes contained relatively low or basal levels of PI kinase activity. We therefore concluded that activation of the NEC receptor was followed by its association with a protein complex that simultaneously contained phospholipase C$_\gamma$ and PI kinase.

Regulatory Subunit of Phosphatidylinositol 3′-Kinase Forms Complex with Ligand-activated Neu Kinase—To directly analyze complex formation between PI 3′-kinase and the *Neu* tyrosine kinase, we raised in rabbits antibodies that were specific to the regulatory subunit of PI 3′-kinase (Carpenter *et al.,* 1990; Morgan *et al.,* 1990; Escobedo *et al.,* 1991; Otsu *et al.,* 1991; Skolnik *et al.,* 1991). As an antigen we used a synthetic peptide corresponding to residues 702–715 of the α-isoform of PI 3′-kinase (p85α) (Otsu *et al.,* 1991). When tested on lysates of murine fibroblasts that express the chimeric receptor (NEC cells), the immune serum could precipitate a phosphatidylinositol kinase activity and an 85-kDa protein (Fig. 6). The preimmune serum was devoid of these activities (Fig. 6). To verify that the protein recognized by our antibody was capable of association with growth factor receptors upon their ligand activation, we chose to examine the interaction with the receptor for PDGF. This receptor is endogenously expressed at high levels in murine fibroblasts such as NEC cells. Using antibodies to the PDGF receptor and p85α, we found that stimulation of NEC cells with PDGF resulted in the formation of a tyrosine-phosphorylated complex between the receptor and p85α (Fig. 7, *upper*). The same

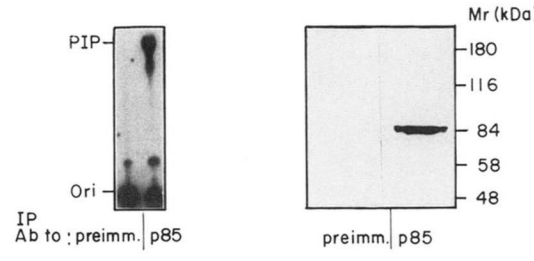

FIG. 6. **Characterization of antibodies to p85α regulatory subunit of PI 3′-kinase.** Lysates of 6 × 10^7 NEC cells were cleared of detergent-insoluble material and subjected to immunoprecipitation (*IP*) with either a preimmune rabbit serum (*preimm.*) or an immune serum as indicated. The latter was raised in rabbits injected with a synthetic peptide corresponding to amino acids 702–715 of the α-isoform of the p85 subunit of PI 3′-kinase (Otsu *et al.,* 1991). Washed immunoprecipitates were either assayed for associated PI kinase activity (*left*) or analyzed by gel electrophoresis and Western blotting (*right*) with the rabbit anti-peptide antiserum. The locations of PIP and the origin (*Ori*) are shown in the autoradiograph of the TLC plate, and the locations of molecular weight marker proteins are indicated on the Western blot. *Ab,* antibody.

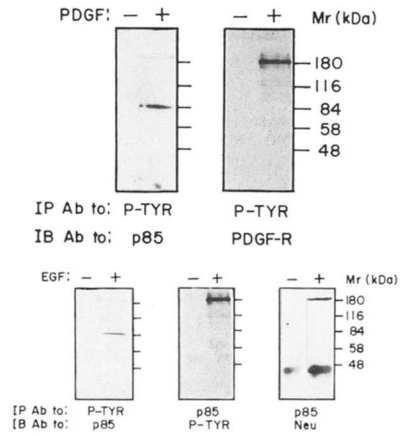

FIG. 7. **Immunoprecipitation of p85-receptor complexes from ligand-stimulated NEC cells.** Serum-starved NEC cells were either untreated (−) or were incubated (+) at 37 °C with 30 ng/ml PDGF (10 min) (*upper*) or with 50 ng/ml EGF (5 min) (*lower*). Cell lysates were then prepared and subjected to immunoprecipitation (*IP*) with either an antibody (*Ab*) to phosphotyrosine (*P-TYR*) or an antibody to p85α. After gel electrophoresis, the immunoprecipitated proteins were electrophoretically transferred onto nitrocellulose and immunoblotted (*IB*) with the indicated antibodies to p85α, the PDGF receptor, phosphotyrosine, or p185^{neu}. The autoradiographs of the Western blots are shown along with the locations of molecular weight marker proteins.

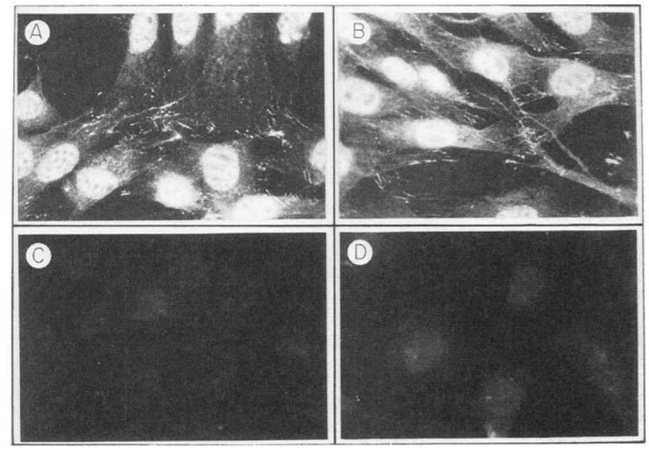

FIG. 8. **Immunofluorescent labeling of p85 in NEC cells.** NEC cells were left untreated (*A*) or were treated (*B–D*) with 50 ng/ml EGF for 1 min at 37 °C. The cells were then fixed with paraformaldehyde, permeabilized with Triton X-100, and incubated with an antibody to p85α (*A–C*) or the preimmune rabbit serum (*D*). As a control, the incubation with the antibodies was performed with the immunizing synthetic peptide (*C*) at a concentration of 0.1 mg/ml. To localize the first antibody, a rhodamine-conjugated goat anti-rabbit antibody was added. Visualization of the fluorescent labeling was done using a Zeiss fluorescent microscope. Photographs of the cells obtained on Kodak X-Omat 400 film are shown. Note the intense labeling of the nuclei, but not the nucleoli, and the decoration of membranes along restricted areas.

analysis of the NEC cells after stimulation with EGF yielded similar results (Fig. 7, *lower*): the NEC protein was coimmunoprecipitated with antibodies to p85α. Although antibodies to phosphotyrosine were able to immunoprecipitate p85α only after stimulation with the ligand, our immunoblotting with antibodies to phosphotyrosine only detected phosphorylation of the NEC protein, with no evidence for direct tyrosine phosphorylation of p85α (Fig. 7, *lower*).

Cellular Distribution of p85α—It has been previously shown that tyrosine phosphorylation of proteins that contain *src* homology domains induces their redistribution from the cytoplasm to membrane sites (see Todderud *et al.* (1990); Cohen *et al.* (1990), and Varticovski *et al.* (1991); reviewed by Koch *et al.* (1991) and Rhee (1991)). To examine the possibility that p85α undergoes translocation following activation of the *Neu* receptor, we used our antibodies to localize the protein in NEC cells before and after the addition of EGF. Immunofluorescent analysis revealed that p85α was localized to two major sites within the cell: the nucleus and confined regions of the plasma membrane (Fig. 8). The specificity of the immunostaining of these sites was implied using the preimmune serum (Fig. 8*D*) or the immune serum in the presence of the immunizing synthetic peptide (Fig. 8*C*). These control analyses showed only basal autofluorescence of the cells. The nuclear distribution of p85α avoided the nucleoli and appeared to be the major cellular site; yet although little or no diffuse cytoplasmic staining was detected, long and narrow regions of the plasma membrane were decorated. Comparison of the fluorescent micrographs obtained from cells stained in the presence or absence of the ligand of NEC revealed no prominent alteration in the pattern of cellular distribution of p85α (Fig. 8). However, it is possible that the sensitivity of our detection method was insufficient to reveal redistribution of the apparently small fraction of p85α that became associated with NEC. Alternatively, cellular sites of localization that escaped the fixation step may exist.

Association of PI Kinase with Mutants of Oncogenic Neu Protein—It has been previously suggested that the oncogenic point-mutated version of the *neu* gene encodes a permanently activated tyrosine kinase that is functionally equivalent to a ligand-occupied receptor (Stern *et al.*, 1988; Yarden, 1990). This led us to examine whether the oncogenic *Neu* protein, in which Val^{664} is replaced by a glutamic acid, is permanently associated with PI kinase activity. Comparison of immunoprecipitates of *Neu* proteins that were prepared from cells that overexpress the normal (NE19 cells) or the transforming (B104-1-1 cells) protein at comparable levels (Fig. 9*B*) revealed that the latter immunocomplexes were characterized by relatively high levels of the lipid kinase (Fig. 9*A*). In contrast, a tyrosine kinase-defective mutant of the oncogenic p185^{neu} in which Lys^{758} is replaced by alanine (Peles *et al.*, 1991) completely lost the ability to associate with PI kinase (Fig. 9*A*). Another mutant of the Glu^{664} version of *Neu* in which the whole carboxyl-terminal tail of 255 amino acids is deleted (ΔCT mutant) also lost the capacity to associate with PI kinase (Fig. 9*C*). This was probably due to the removal of all the known tyrosine phosphorylation sites (Hazan *et al.*, 1989) as no phosphotyrosine could be detected in this protein in living cells (Fig. 9*B*). Both the defective kinase and the carboxyl-terminally deleted mutant of the oncogenic *Neu* protein also lost their ability to induce a transformed phenotype on the basis of the following criteria: foci formation on a monolayer of Rat-1 fibroblasts, soft agar colony formation assay, and the induction of tumorigenic growth in athymic mice.[2] It was therefore concluded that oncogenic activation of the *Neu* protein is accompanied by a noncovalent association with PI kinase, but mutations that affect the tyrosine kinase (K758A) or all the phosphorylation sites (ΔCT) abolish this permanent coupling.

DISCUSSION

Our present study demonstrates that phosphatidylinositol 3′-kinase undergoes rapid association with a ligand-activated *Neu* tyrosine kinase and that the permanently activated transforming p185^{neu}, but not its nontransforming variants, is constitutively coupled to PI 3′-kinase. These observations not only add the ligand of p185^{neu} to the list of growth factors

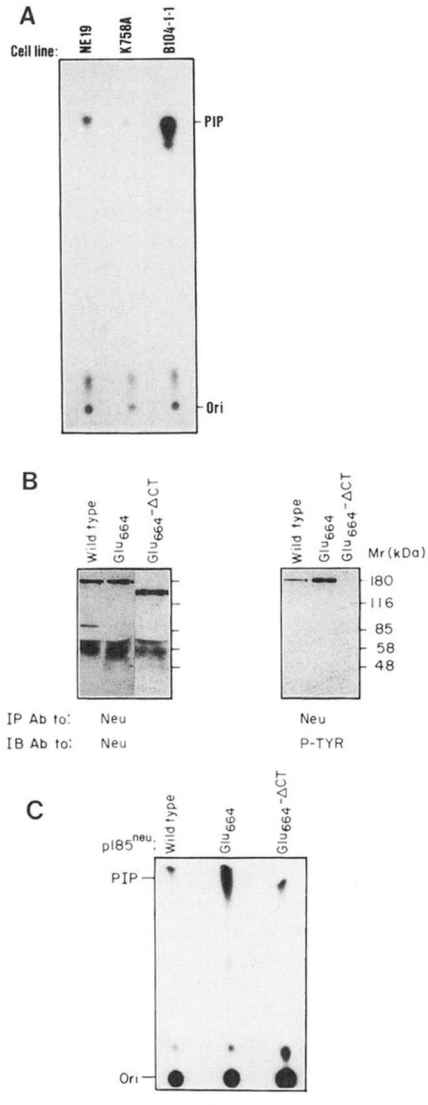

FIG. 9. **Association of PI kinase activity with mutants of *Neu* protein.** Cell lysates were prepared from confluent monolayers of the following cell lines: NE19 cells, which express the wild-type *Neu* protein (Val^{664}); B104-1-1 cells, which express the transforming $p185^{neu}$ (Glu^{664}); or K758A cells, which express a kinase-defective double mutant of *Neu* (Glu^{664} mutant with Lys^{758} replaced by Ala). A cell line expressing a carboxyl-terminally deleted *Neu* protein lacking 255 amino acids was also used (ΔCT). The *Neu* proteins were immunoprecipitated (*IP*) with a mixture of three monoclonal antibodies (*Ab*) (Yarden, 1990). The immunocomplexes were then tested for associated PI kinase activity by an *in vitro* kinase assay (*A* and *C*) or analyzed by immunoblotting (*IB*) with antibodies to *Neu* (NCT antibody) or antibodies to phosphotyrosine (*P-TYR*) as indicated (*B*). *Ori*, origin.

that mediate coupling to PI 3′-kinase (*e.g.* PDGF (Kaplan *et al.*, 1987), EGF (Bjorge *et al.*, 1990), colony-stimulating factor 1 (Varticovski *et al.*, 1989), stem cell factor (Lev *et al.*, 1991), and insulin (Ruderman *et al.*, 1990)), but also adds the *neu* oncogene to the group of transforming proteins that apparently utilize PI 3′-kinase as an effector enzyme (*e.g.* polyoma middle T (Courtneidge and Heber, 1987), *Src* (Fukui and Hanafusa, 1989; Fukui *et al.*, 1989), *abl* (Varticovski *et al.*, 1991), and *Ros* (Macara *et al.*, 1984)). Our results are consistent with the results of Scott *et al.* (1991), who found that activation of the tyrosine kinase of the human $p185^{neu}$, by means of a monoclonal antibody, induces a 2-fold increase in inositol-1,4,5-triphosphate 3′-kinase activity. In addition, the coupling between $p185^{neu}$ and PI 3′-kinase is reminiscent of the ligand-induced association of the EGF receptor with the lipid kinase (Bjorge *et al.*, 1990) and underscores the functional similarity between these homologous receptors.

The identification of the *Neu*-associated PI kinase as a type I enzyme was based on its susceptibility to inhibition by Nonidet P-40, chromatographic separation of the phosphorylated product from phosphatidylinositol 4-phosphate, and detection of the regulatory subunit of the enzyme (p85α) using specific antibodies. In addition, the data indicate that PI 3′-kinase is the major PI kinase that associates with $p185^{neu}$ in a ligand-dependent manner (Fig. 4); yet we cannot exclude a minor contribution by the type II kinase that was found to associate, under certain conditions, with the human $p185^{neu}$ (Scott *et al.*, 1991) and the EGF receptor (Cochet *et al.*, 1991). Despite the observation that several proteins underwent phosphorylation on tyrosine residues upon ligand stimulation of the tyrosine kinase of *Neu* (Fig. 1), we have not been able to directly demonstrate that p85α underwent modification of tyrosine residues, as was the case in other systems (reviewed by Cantley *et al.* (1991)); yet PI 3′-kinase activity and p85α were precipitable with antibodies to phosphotyrosine (Figs. 5 and 7), but only after ligand stimulation. Thus, either p85α was part of a tyrosine-phosphorylated complex but did not undergo tyrosine phosphorylation by itself, or the fraction of cellular p85α that was modified by the activated receptor was too low to be detected by our Western blot analysis. We recently addressed this question by Western blotting of p85α in large-scale immunoprecipitates of the stimulated NEC receptor (data not shown). This analysis revealed that only 0.12% of cellular p85α associated with the NEC protein after 2 min of ligand stimulation at 37 °C. This low proportion is consistent with our inability to detect significant cellular translocation of p85α by immunofluorescence (Fig. 8) and may explain the lack of tyrosine phosphorylation signal in p85α. Interestingly, however, we reproducibly recovered 10–15% of the total cellular PI 3′-kinase activity in receptor immunoprecipitates. This observation may indicate that the association of the lipid kinase with the *Neu* protein results in at least 10-fold activation of the catalytic activity of PI 3′-kinase.

Our results (Fig. 1), as well as those of other laboratories (Peles *et al.*, 1991; Fazioli *et al.*, 1991; Scott *et al.*, 1991), indicate that in addition to PI 3′-kinase, phospholipase C_γ and GTPase-activating protein also form a complex with the activated *Neu* protein. Initial characterization of this multiprotein aggregate suggested that phospholipase C_γ and PI 3′-kinase molecules were simultaneously associated with the tyrosine-phosphorylated receptor (Fig. 5), but we have so far failed to detect similar coprecipitation of PI kinase activity by antibodies to GTPase-activating protein, Raf-1, and the p36 (calpactin) major substrate of $pp60^{src}$ (data not shown). By analogy, a simultaneous complex of the PDGF receptor with Raf-1, phospholipase C_γ, PI 3′-kinase, and GTPase-activating protein has been reported (Kaplan *et al.*, 1990).

While looking for cellular redistribution of p85α following activation of $p185^{neu}$, we were surprised to find that the regulatory subunit was almost exclusively localized in restricted sites on the plasma membrane and in the nucleus and that this distribution was not affected by ligand binding (Fig. 8). Consistent with the immunocytochemical localization, biochemical analysis recently detected PI kinase activity in the cytoskeleton and perhaps also the nuclear matrix of cells extracted with Triton X-100 (Payrastre *et al.*, 1991). We currently are investigating the membrane site of localization of p85α using antibodies to various cytoskeletal proteins. It is relevant that phospholipase C_γ, a protein that shares with

p85α related *src* homology (SH) domains, displayed a similar distribution at membrane or cytoskeletal sites (McBride *et al.*, 1991). The association of p85α with the plasma membrane could occur through its SH2 domains (anchored to cytoskeletal phosphotyrosine proteins); or it could be mediated by the SH3 domain, which is thought to act as an actin-binding site (Drubin *et al.*, 1990). How this localization relates to the function of PI 3′-kinase is an open question. Similarly, the localization to the nucleus, but not to the nucleoli, raises interesting questions. For example, p85α may act as an adaptor protein to regulate enzymes other than the catalytic subunit of PI 3′-kinase. It is relevant that according to a recent report, the polyphosphoinositide cycle exists in the cell nucleus, as does protein kinase C (Divecha *et al.*, 1991).

The invariable coupling of all the tyrosine kinase oncoproteins, including $p185^{neu}$ with the PI 3′-kinase (reviewed by Carpenter and Cantley (1990)), underscores the still unknown function of this lipid kinase in the regulation of cell growth. In this respect, our observation that the transforming $p185^{neu}$, but not three nontransforming *Neu* proteins, strongly associates with PI 3′-kinase is consistent with similar observations made with mutants of polyoma middle T (Courtneidge and Heber, 1987) and $pp60^{v\text{-}src}$ (Fukui and Hanafusa, 1989). However, the association of PI 3′-kinase with the *Neu* protein appears to depend not only on the level of tyrosine kinase activity, but also on the existence of tyrosine phosphorylation sites on the receptor. This conclusion was illustrated by the lack of association with a kinase-defective $p185^{neu}$ and also by another Glu^{664} mutant in which the kinase domain was intact but all the phosphorylation sites were deleted (ΔCT). Nevertheless, even in the presence of an intact kinase domain and autophosphorylation sites, dissociation eventually takes place (Fig. 3, *lower*), even though the high level of tyrosine phosphorylation of the receptor persists under these conditions (data not shown). Apparently, additional molecular mechanisms control the formation and breakdown of the $p185^{neu}$-PI 3′-kinase complex. Further characterization of the interactions that affect the identity and stability of the signaling molecules utilized by $p185^{neu}$ is expected to shed light on the involvement of specific effector proteins in the mechanism of signal transduction by normal and oncogenic forms of $p185^{neu}$.

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