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ErbB3 Is Involved in Activation of Phosphatidylinositol 3-Kinase by Epidermal Growth Factor

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Conflicting results concerning the ability of the epidermal growth factor (EGF) receptor to associate with and/or activate phosphatidylinositol (PtdIns) 3-kinase have been published. Despite the ability of EGF to stimulate the production of PtdIns 3-kinase products and to cause the appearance of PtdIns 3-kinase activity in antiphosphotyrosine immunoprecipitates in several cell lines, we did not detect EGF-stimulated PtdIns 3-kinase activity in anti-EGF receptor immunoprecipitates. This result is consistent with the lack of a phosphorylated Tyr-X-X-Met motif, the p85 Src homology 2 (SH2) domain recognition sequence, in this receptor sequence. The EGF receptor homolog, ErbB2 protein, also lacks this motif. However, the ErbB3 protein has seven repeats of the Tyr-X-X-Met motif in the carboxy-terminal unique domain. Here we show that in A431 cells, which express both the EGF receptor and ErbB3, PtdIns 3-kinase coprecipitates with the ErbB3 protein ($p180^{erbB3}$) in response to EGF. $p180^{erbB3}$ is also shown to be tyrosine phosphorylated in response to EGF. In contrast, a different mechanism for the activation of PtdIns 3-kinase in response to EGF occurs in certain cells (PC12 and A549 cells). Thus, we show for the first time that ErbB3 can mediate EGF responses in cells expressing both ErbB3 and the EGF receptor.

Phosphatidylinositol (PtdIns) 3-kinase is a cytosolic protein that is recruited to activated protein tyrosine kinases in response to a variety of factors that activate cells. It is a heterodimeric protein consisting of 85- and 110-kDa subunits (4, 26). The 85-kDa subunit (p85) contains two Src homology 2 (SH2) groups that bind to tyrosine-phosphorylated amino acids that have a consensus Tyr-X-X-Met motif. The 110-kDa subunit contains the catalytic site (13). PtdIns 3-kinase is activated by various growth factors, including epidermal growth factor (EGF), platelet-derived growth factor, colony-stimulating factor 1, and insulin (reviewed in reference 3). An increase in PtdIns 3-kinase activity also occurs in response to nerve growth factor (6, 35, 38), a factor that initiates cellular differentiation. PtdIns 3-kinase activity is also activated by addition of fMet-Leu-Phe to neutrophils (43) and thrombin to platelets (21, 25). PtdIns 3-kinase has been shown to bind directly to activated receptors at domains that are autophosphorylated on tyrosine and contain the Tyr-X-X-Met motif (3). The activation of PtdIns 3-kinase by insulin involves the association of p85 with an intermediary cytosolic protein, IRS-1, that is tyrosine phosphorylated in response to insulin and contains multiple Tyr-X-X-Met motifs (41). The activation of B lymphocytes by antigen binding to tyrosine-phosphorylated membrane immunoglobulin M results in the recruitment of PtdIns 3-kinase activity to tyrosine-phosphorylated CD19, a membrane protein that can be coligated with membrane immunoglobulin M but which does not have intrinsic protein-tyrosine kinase activity (44). Thus, in some cases the activation of PtdIns 3-kinase involves its association with a tyrosine kinase substrate that is distinct from the receptor to which the activating factor binds.

The precise role of the EGF receptor (EGFR) in the activation of PtdIns 3-kinase by EGF remains unclear. EGF produces various effects on cells (reviewed in reference 5). EGF stimulates the canonical PtdIns turnover pathway, which involves the phospholipase C-γ1-mediated hydrolysis of PtdIns 4,5-bisphosphate into inositol-1,4,5-trisphosphate (Ins-1,4,5-P_3) and diacylglycerol. Consistent with this, EGF has been reported to phosphorylate phospholipase C-γ1 (22, 23, 45), increase Ins-1,4,5-P_3 production (12, 31), release Ca^{2+} from intracellular stores and elevate intracellular free Ca^{2+} concentration (15), and activate Ca^{2+}-sensitive ion channels (29). In A431 cells, phosphoinositide kinase activities were found to copurify with the EGFR (42), and EGF stimulated a PtdIns kinase activity that was independent from the production of Ins-1,4,5-P_3 (31). Subsequently, PtdIns 4-kinase and PtdIns-4-P 5-kinase activities, enzymes in the canonical phosphoinositol turnover pathway, were shown to be stimulated by EGF and to associate with the EGFR (7, 27). However, EGF also has been shown to stimulate the appearance of lipid products of PtdIns 3-kinase in a Leydig tumor cell line (30) and in PC12 cells (6, 35). EGF-stimulated PtdIns 3-kinase activity was immunoprecipitated with anti-P-Tyr (6, 24, 35) and anti-EGFR (2) antibodies. However, the EGFR lacks a tyrosine-phosphorylated Tyr-X-X-Met motif and binds p85 very weakly compared with the platelet-derived growth factor receptor (14).

A number of proteins related to the EGFR make up an extended family of type I receptor tyrosine kinases that share sequence homologies that include two cysteine-rich extracellular domains, a single membrane-spanning transmembrane domain, and a cytoplasmic domain that has tyrosine kinase homology. In addition to the EGFR, other family members include ErbB2 ($p185^{erbB2/neu}$) (reviewed in reference 47), ErbB3 ($p180^{erbB3}$) (20, 33), and ErbB4 ($p180^{erbB4}$) (32). ErbB3 is of particular interest in regard to signalling since it contains seven copies of the Tyr-X-X-Met motif in its carboxy-terminal cytosolic domain (39). This observation and reports that ErbB2 may be a substrate for the EGFR (16, 18) suggest that the EGFR interacts with other similar proteins in this class. Thus,

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we hypothesized that EGF activates PtdIns 3-kinase by an interaction between EGFR and ErbB3.

In these studies we compared the effects of EGF on three different cell lines. In all three cell lines, there is an increase in anti-P-Tyr-immunoprecipitable PtdIns 3-kinase activity in EGF-treated cells, but this is not observed in immunoprecipitates collected with anti-EGFR antibody. However, there is an increase in anti-ErbB3-immunoprecipitable PtdIns 3-kinase activity in EGF-treated A431 cells but not in PC12 or A549 cells. Tyrosine-phosphorylated ErbB3 associated with anti-PtdIns 3-kinase (αp85) antibody and recombinant p85 in EGF-treated A431 cells but not in the other cells. Our studies indicate that EGF stimulates the association of PtdIns 3-kinase with ErbB3 in A431 cells but not in PC12 cells or A549 cells.

MATERIALS AND METHODS

Chemicals. All chemicals were reagent grade or better. [^{32}P]ATP (specific activity, 3,000 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.). Dulbecco's modified Eagle medium was obtained from GIBCO Laboratories. EGF (catalog number 01-107) was purchased from Upstate Biochemicals Inc. (Lake Placid, N.Y.).

Antibodies. The anti-phosphotyrosine antibody (anti-P-Tyr) was a murine monoclonal antibody and was generously supplied by Brian Drucker (Dana-Farber Cancer Institute, Boston, Mass.). Antibody (anti-p85) to the 85-kDa subunit of PtdIns 3-kinase was raised in rabbits by Brian Schauffhausen (Tufts University, Boston, Mass.) and is commercially available from Upstate Biochemicals (catalog number 06-195). This is a 1:1 mixture of rabbit antibodies directed against a glutathione *S*-transferase (GST) fusion protein containing the N-terminal SH2 (NSH2) domain of the p85 subunit of PtdIns 3-kinase and against a GST fusion protein containing the full-length p85 subunit. When noted, some experiments were performed with the antibody raised against the NSH2 domain. The following mouse monoclonal anti-EGFR antibodies were used: 13A9 (Genentech, South San Francisco, Calif.), 291-3A and 291-4A (Randy Schatzman, Syntex), clone 29.1 (Sigma Chemical Co., St. Louis, Mo.), and SC-120 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). Anti-ErbB3 antibodies 49-3 (34) and 3183 were raised in rabbits to a synthetic peptide containing cytoplasmic sequence (ELEPELDLDLDLE), and 61-3 (34) and 3185 were raised to a peptide sequence (AMRRYLERGES IE) from the juxtamembrane domain; these antibodies were affinity purified. A rabbit polyclonal antibody to the extracellular domain of human breast ErbB3 was purchased from Transduction Laboratories (Lexington, Ky.). The anti-Neu (Randy Schatzman, Syntex) and K1 anti-ErbB3 (John Koland, University of Iowa) antibodies were raised in rabbits.

Cell culture. A431 cells and A549 cells were obtained from the American Type Culture Collection, and PC12 cells were obtained from Larry Feig (Tufts University). Unless stated otherwise, cells used in lipid kinase assays were grown in 100-mm-diameter dishes, and cells used in Western blotting (immunoblotting) were grown in 150-mm-diameter dishes. All cells were grown in Dulbecco's modified Eagle medium plus serum at 37°C in a 95% air–5% CO_2 mixture. PC12 cells were grown in 5% horse serum plus 5% calf serum, and A431 cells and A549 cells were grown in 10% fetal bovine serum. All cells were used when confluent or nearly confluent. Where noted, cells were switched to serum-free medium (Dulbecco's modified Eagle medium plus 0.1% bovine serum albumin [BSA]) overnight prior to exposure to growth factors.

Assay of PtdIns 3-kinase activity. Growth factors were added for the indicated times at 37°C to cells that were placed in serum-free medium overnight. The cells were washed twice with ice-cold buffer A (137 mM NaCl, 20 mM Tris, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2 mM vanadate [pH 7.5]), and were lysed in lysis buffer (buffer A plus 10% [vol/vol] glycerol, 1% [vol/vol] Nonidet P-40 [NP-40], and 1 mM phenylmethylsulfonyl fluoride). The lysates were vortexed and centrifuged at 16,000 × *g* (Eppendorf 5414 microcentrifuge). The cleared supernatants were transferred to fresh microcentrifuge tubes and incubated with anti-P-Tyr (6.6 μg/ml) or other antibodies (see figure legends) for 2 h at 4°C. Protein A-Sepharose (4 mg/ml of lysate) was then added to the lysates for 2 h at 4°C. In some experiments, antibodies and protein A-Sepharose were added simultaneously to the lysates for 3 h.

The immunoprecipitates were pelleted, washed three times in phosphate-buffered saline (PBS) (137 mM NaCl, 15.7 mM NaH_2PO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl [pH 7.4])–1% NP-40, two times in 0.1 M Tris (pH 7.5)–0.5 M LiCl, and two times in TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA [pH 7.5]). All wash solutions contained 200 μM vanadate. To assay the PtdIns 3-kinase activity of the immunoprecipitates, sonicated PtdIns [0.2 mg/ml (final concentration) in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (pH 7.5)] and [γ-^{32}P]ATP (10 to 20 μCi per sample) were added to the immunoprecipitates for 10 min at room temperature. When noted, 0.5% (vol/vol) NP-40 also was present in the reaction mixture. The reaction was stopped by the addition of 80 μl of 1 M HCl and 160 μl of methanol-chloroform (1:1 [vol/vol] mixture). The lipid-containing organic phase was resolved on oxalate-coated thin-layer chromatography plates (Silica Gel 60; MCB Reagents, Merck, Rahway, N.J.) developed in chloroform-methanol-water-ammonium hydroxide (60:40:11.3:2, vol/vol). Radiolabelled spots corresponding to authentic PtdIns-4-P were excised and quantified by scintillation counting or Cerenkov radiation. In some experiments, the PtdIns phosphate (PtdInsP) spot was deacylated and subjected to high-pressure liquid chromatography (HPLC) analysis to determine lipid identity and quantification, as previously described (1).

Identification of proteins by Western blot assays. Lysates from confluent or nearly confluent cells grown in a 150-mm-diameter culture dish were prepared as described above. Cleared lysates were immunoprecipitated with various antibodies plus protein A-Sepharose for 3 to 5 h at 4°C. Antibodies were used as designated in figure legends. The Sepharose beads were pelleted, washed (three times with PBS–1% NP-40, twice with 0.1 M Tris–0.5 M LiCl, and twice with TNE), diluted with an equivalent volume of 2× sample buffer, and boiled for 5 min. The supernatant was subjected to electrophoresis on a sodium dodecyl sulfate (SDS)–7% polyacrylamide separating gel with a 3% stacking gel. Proteins were transferred to 0.2-μm-pore-size nitrocellulose filters, and the filters were blocked with TBS (20 mM Tris, 137 mM NaCl [pH 7.6])–2% BSA for 1 h. The filters were washed in TBS–0.2% Tween 20 (TTBS) three times. To identify tyrosine-phosphorylated proteins, the blots were exposed to anti-P-Tyr (1 μg/ml) in TTBS–1% BSA for 16 h at 4°C. The identification of proteins with other antibodies is as reported in figure legends. The filters were washed three times in TTBS and exposed to secondary antibody (anti-mouse horseradish peroxidase or anti-rabbit horseradish peroxidase [Boehringer-Mannheim]) at a 1:10,000 dilution in TTBS–1% BSA for 1 h. Filters were washed three times with TTBS and twice with TBS and were visualized with a chemiluminescence system (ECL, Amersham). In some experiments, the filters were stripped by exposing them to 62.5 mM Tris (pH 6.8)–β-mercaptoethanol

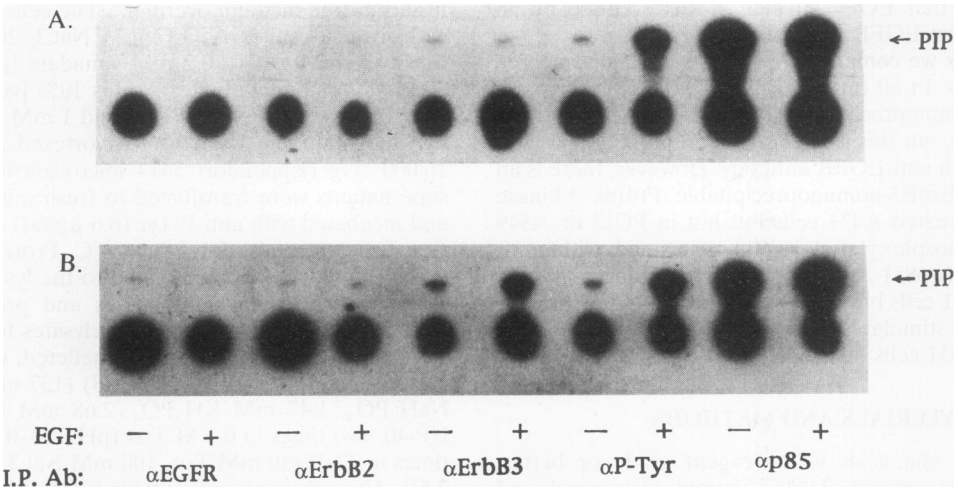

FIG. 1. Immunoprecipitation of PtdIns 3-kinase activity from EGF-stimulated PC12 and A431 cells. Serum-starved PC12 cells (A) or A431 cells (B) were left untreated (−) or were treated for 5 min with EGF (100 ng/ml) (+). Lysates were prepared in 1 ml of lysis buffer, and PtdIns 3-kinase activity was measured in immunoprecipitates (I.P.) of the cleared lysates (see Materials and Methods). PtdIns 3-kinase activity was measured by using exogenous PtdIns as a substrate. The following amounts of antibodies (Ab) were added to 1 ml of cleared lysate: anti-EGFR (29.1), 5 μl; anti-ErbB2, 5 μl; anti-ErbB3 (K1), 5 μl; anti-P-Tyr, 2 μg/ml; and anti-p85, 2 μl. The dark area below the PtdInsP (PIP) spot is radioactivity at the origin and represents ATP or related products.

(0.1 M)–2% SDS at 70°C for 30 min. The stripped filters were washed several times in TBS, blocked with TBS–2% BSA, and reprobed with other antibodies as described above or in figure legends.

Expression of proteins in insect cells. The baculovirus expression system was used to infect Sf9 insect cells with cDNAs encoding human EGFR and bovine $p180^{erbB3}$. This will be described in a forthcoming paper (10a).

GST fusion proteins. GST fusion proteins of the intact p85 were produced as described previously (48).

Data. Data are given as means ± standard errors of the mean, with the number of determinations (n) representing separate experiments.

RESULTS

Effects of EGF on PtdIns 3-kinase activity in anti-P-Tyr immunoprecipitates. Since the association of PtdIns 3-kinase activity with the EGFR has been controversial (see Introduction), we examined the activation of PtdIns 3-kinase in anti-P-Tyr immunoprecipitates of several cell lines that responded to EGF. After a 5-min exposure to EGF (100 ng/ml), the PtdIns 3-kinase activity was increased to 11.2 ± 2.4 (n = 16) and 37.2 ± 4.3 (n = 24) times the basal (unstimulated) activity in A431 and PC12 cells, respectively. To confirm that the product of the PtdIns kinase assay was PtdIns-3-P, the product of PtdIns 3-kinase, the PtdInsP identified via thin-layer chromatography was deacylated and analyzed by HPLC. In growth factor-treated cells, PtdIns-3-P made up more than 85% of the PtdInsP radioactivity (not shown).

Association of PtdIns 3-kinase activity with EGFR, ErbB2, and ErbB3. To examine whether the enhanced PtdIns 3-kinase activity in EGF-treated cells involved the association of PtdIns 3-kinase with the EGFR or with other members of the type I (EGFR-like) family of growth factor receptors, we measured PtdIns 3-kinase activity in immunoprecipitates by using antibodies to the EGFR (ErbB1), ErbB2, and ErbB3 proteins, as well as with anti-P-Tyr and anti-p85 antibodies. For each experiment with a particular cell line, the PtdIns 3-kinase assays were performed at the same time with cells of the same passage to facilitate a direct comparison of these five antibodies. In PC12 cells, EGF-stimulated PtdIns 3-kinase activity was readily detected in anti-P-Tyr immunoprecipitates, but not in anti-EGFR (29.1 or 13A9), anti-ErbB2, or anti-ErbB3 (K1) immunoprecipitates (Fig. 1A and Table 1). In contrast, in A431 cells a significant amount of EGF-stimulated PtdIns 3-kinase activity was found in anti-ErbB3 (K1) immunoprecipitates as well as in anti-P-Tyr immunoprecipitates, but not in anti-EGFR (29.1) or anti-ErbB2 immunoprecipitates (Fig. 1B and Table 1). Treatment of A431 cells with EGF increased the lipid kinase activity in anti-ErbB3 (K1) immunoprecipitates to 6.2 ± 1.0 (n = 7) times the basal level. Increases in immunoprecipitable PtdIns 3-kinase activity from EGF-treated A431 cells were also found with other anti-ErbB3 antibodies, as follows: 61-3, 5.3 ± 2.1 (n = 4) times the basal activity; 3183, 3.2 ± 0.6 (n = 7); 3185, 3.1 ± 1.1 (n = 3); and Transduction, 5.5 ± 1.3 (n = 3). Notably, in experiments in which both anti-ErbB3 and anti-P-Tyr immunoprecipitates were examined at the same time, the amount of EGF-stimulated PtdIns 3-kinase activity in the anti-ErbB3 immunoprecipitates was 49.5% ± 24.7% (n = 5, with K1). The activity in anti-ErbB3 (K1) immunoprecipitates was blocked by >95% (n = 2) by the presence of 0.5% NP-40, which inhibits PtdIns 3-kinase activity (46) in the lipid kinase assay. These results suggest that ErbB3 is involved in the EGF-promoted activation of PtdIns 3-kinase in A431 cells but not in PC12 cells and that tyrosine phosphorylation was involved in the activation.

Since other investigators had reported the association of PtdIns 3-kinase activity with the EGFR, we examined several other EGFR antibodies for their abilities to immunoprecipitate EGF-stimulated PtdIns 3-kinase activity from A431 cells. First, we compared the abilities of different antibodies to immunoprecipitate the EGFR. Three antibodies (29.1, 13A9, and SC-120) immunoprecipitated relatively greater amounts of EGFR from A431 cells compared with two other antibodies (291-3A and 291-4A) (Fig. 2A). However, no EGF-stimulated PtdIns kinase activity was measured in immunoprecipitates obtained with these three antibodies (Fig. 2B). In these

TABLE 1. Immunoprecipitation of PtdIns 3-kinase activity with antibodies to different type I receptor tyrosine kinases

Cells and antibody	Presence of EGF	No. of cpm[a]	
		Expt 1	Expt 2
A431			
αEGFR	−	43	454
	+	41	604
αErbB2	−	49	580
	+	68	510
αErbB3	−	180	1,390
	+	1,589	7,903
αP-Tyr	−	141	1,670
	+	3,849	6,198
αp85 (NSH2)	−	16,487	352,828
	+	22,489	389,534
PC12			
αEGFR	−	26	26*
	+	36	27*
αErbB2	−	28	7*
	+	34	32*
αErbB3	−	67	90*
	+	48	63*
αP-Tyr	−	101	101*
	+	3,528	4,337*
αp85 (NSH2)	−	43,186	115,583*
	+	70,756	83,464*

[a] All values are Cerenkov counts, except those marked by an asterisk, which are scintillation counts.

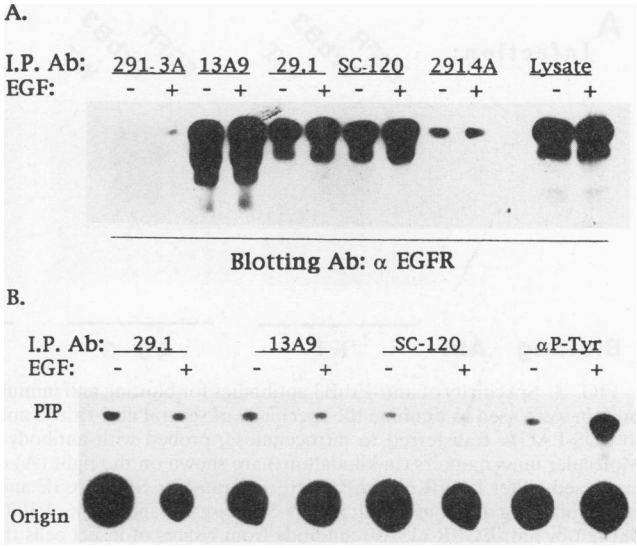

FIG. 2. Relative effects of different anti-EGFR antibodies in immunoprecipitating EGFR (A) or PtdIns kinase (B) activity from A431 cells. Cells were serum starved overnight and left untreated (−) or treated with EGF (100 ng/ml) for 5 min (+). (A) Lysates of cells grown in 100-mm-diameter dishes were prepared in 1 ml of lysis buffer, and the following antibodies (Ab) were added to part (400 μl) of the cleared lysates: 291-3A (3 μl), 13A9 (2 μl), 29.1 (3 μl), SC-120 (3 μl), and 291-4A (3 μl). The immunoprecipitates (I.P.) were washed, subjected to SDS-PAGE, transferred to nitrocellulose filters, and probed overnight at 4°C with anti-EGFR (291-3A, 1:400 dilution). A portion (40 μl) of the cleared lysate was also subjected to SDS-PAGE. (B) PtdIns kinase assays were performed as previously described (see Materials and Methods) on immunoprecipitates from A431 cells. The following antibodies were added to 1 ml of cleared lysate: 29.1 (3 μl), 13A9 (2 μl), SC-120 (3 μl), and anti-P-Tyr (6.6 μg/ml). PIP, PtdInsP.

experiments, we extracted the PtdInsP spots from the thin-layer chromatography and subjected them to HPLC analysis to confirm that PtdIns-3-P did not increase in response to EGF in the anti-EGFR immunoprecipitates (not shown).

Since A431 cells are a human cell line and PC12 cells are derived from rat cells, we examined another human cell line to determine if PtdIns 3-kinase activity was also present in anti-ErbB3 immunoprecipitates. A549 cells are derived from a human lung carcinoma. Treatment of these cells with EGF (100 ng/ml, for 5 min) increased the anti-P-Tyr immunoprecipitable PtdIns 3-kinase activity to 15.7 ± 5.8 (n = 3) times that of unstimulated cells, but EGF did not produce an increase in anti-ErbB3 (K1) immunoprecipitates or in anti-EGFR (29.1 or 13A9) immunoprecipitates (data not shown).

As indicated in Table 1 and in other experiments, the PtdIns 3-kinase activity of anti-P-Tyr immunoprecipitates of growth factor-treated A431 and PC12 cells was much less (generally less than 10%) than the PtdIns 3-kinase activity that was immunoprecipitated with anti-p85. These results indicate that a large amount of PtdIns 3-kinase activity does not associate with receptors or is not tyrosine phosphorylated.

Association of ErbB3 with GST85 and its phosphorylation on tyrosine. The ability of different anti-ErbB3 antibodies to blot ErbB3 expressed in insect cells is shown in Fig. 3A. Three antibodies (K1, 49-3, and SC-06) identified a major band at 170 to 175 kDa and a smaller band at 155 to 160 kDa. Two other antibodies, 3183 and 3185, identified the same bands (not shown). These proteins were not observed in wild-type insect cells or in insect cells that expressed the EGFR. ErbB3 expressed in insect cells was immunoprecipitated with anti-ErbB3 antibody (3185 and 61-3) but not anti-EGFR antibody (13A9) (Fig. 3B).

Initial attempts to detect ErbB3 in A431 cells by immunoblotting failed because of the low abundance of this protein. However, ErbB3 could be detected in association with exogenously added recombinant p85. In these studies, we compared the effects of EGF and insulin, both of which have been found to promote the phosphorylation of a number of proteins on tyrosine. Lysates of EGF- and insulin-treated A431, PC12, and A549 cells were probed with a GST fusion protein (GST85) of the p85 subunit of PtdIns 3-kinase. As a control, cells were also probed with GST alone. The precipitates were washed under the stringent conditions, including high salt concentration, that were used in lipid kinase assays (see Materials and Methods). The precipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) transferred to nitrocellulose, and blotted with anti-ErbB3 (49-3) antibody. Two proteins, one at ~180 kDa and one at ~130 kDa, were observed in GST85 precipitates from A431 cells exposed to EGF (Fig. 4A). These proteins were not seen in parallel experiments conducted with PC12 cells or A549 cells probed with GST85 or in experiments with any of the cell lines probed with GST alone (Fig. 4A). Since anti-ErbB3-immunoprecipitable PtdIns 3-kinase activity was found in A431 cells—but not in PC12 or A549 cells—treated with EGF, these results suggest that there is an association between ErbB3 and p85 in A431 cells but not in the two other cell lines. The ~180-kDa size of the protein

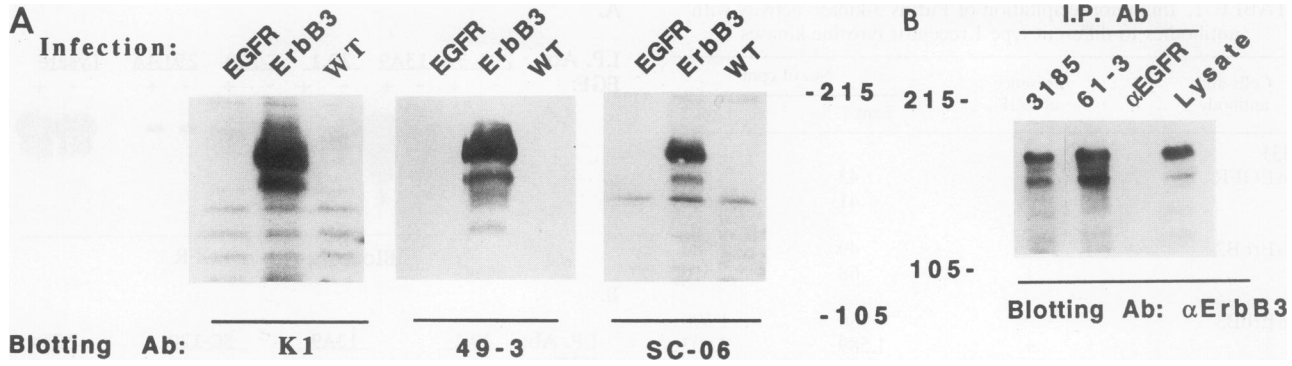

FIG. 3. Specificity of anti-ErbB3 antibodies for blotting and immunoprecipitation. Lysates from Sf9 insect cells that expressed EGFR or ErbB3 protein were used to examine the specificity of several anti-ErbB3 antibodies (Ab) for blotting and immunoprecipitation. Proteins were subjected to SDS-PAGE, transferred to nitrocellulose, probed with antibody overnight, and visualized on X-ray film by enhanced chemiluminescence. Molecular mass markers (in kilodaltons) are shown on the right (A) or left (B). (A) Lysates from wild-type (WT) insect cells and insect cells that expressed either EGFR or ErbB3 were separated by SDS-PAGE and probed overnight at 4°C with one of three different anti-ErbB3 antibodies at the following dilutions: K1, 1:500; 49-3, 0.8 μg/ml; and SC-06, 1:100. (B) ErbB3 was immunoprecipitated by anti-ErbB3 antibodies 3185 and 61-3 but not by anti-EGFR (13A9) antibody from lysates of insect cells that expressed ErbB3. Also shown is lysate alone. Proteins were probed with 3183 (1:1,000). The bands visualized by 3183 were not observed in immunoprecipitations (I.P.) from wild-type insect cells or insect cells infected with EGFR (not shown).

identified by anti-ErbB3 antibody is the same size as ErbB3 found in human mammary tumor cell lines (19) and recombinant ErbB3 expressed in fibroblasts (5a, 19).

When the protein blots were stripped and reprobed with anti-EGFR antibody, a protein identifiable as the EGFR was found at ~170 kDa in GST85 precipitates of EGF-treated cells for all three of the cell lines (Fig. 4B). A431 cells contained the highest relative amount of EGFR identified in the GST85 precipitates. The EGFR band at ~170 kDa was clearly distinguishable from the 180-kDa protein identified with the anti-ErbB3 antibody. The EGFR was not found in lysates probed with GST alone. When the blots were stripped and reprobed with anti-P-Tyr antibody, bands that colocalized with the ErbB3 protein at ~180 kDa in A431 cells and the EGFR protein at ~170 kDa in all three cell lines were found to be tyrosine phosphorylated in response to EGF (Fig. 4C). These results demonstrate the presence of ErbB3 in A431 cells and indicate that treatment of these cells with EGF alters ErbB3 to enhance its ability to associate with p85 in vitro. Although only ErbB3 was found to associate with PtdIns 3-kinase in vivo (Fig. 1), both ErbB3 and EGFR associated with p85 in vitro.

ErbB3 and other tyrosine-phosphorylated proteins are immunoprecipitated with anti-p85 antibody. Additional evidence for the association of PtdIns 3-kinase with the ErbB3 protein in A431 cells was obtained with anti-p85 antibody. An ~180-kDa protein was identified as ErbB3 (Fig. 5A) in immunoblots of anti-p85 immunoprecipitates from EGF-treated cells, and tyrosine phosphorylation of the ~180-kDa protein was greatly increased in cells treated with EGF (Fig. 5B). The tyrosine phosphorylation of ErbB3 was increased within 30 s of exposure of A431 cells to EGF, and enhanced phosphorylation was observable for at least 15 min (Fig. 6). In these immunoprecipitates there was an increase in the tyrosine phosphorylation of several proteins at molecular masses similar to those found after precipitation of A431 cells with the GST85 probe (Fig. 4). The bands at ~180 and ~130 kDa in the anti-p85 immunoprecipitate of A431 cells (Fig. 6) are consistent with the bands identified in GST85 precipitates by using anti-ErbB3 antibody (Fig. 4A). A light band at ~170 kDa may be the EGFR, although this receptor could not be detected by immunoblot (not shown). The ~130- and ~170-kDa tyrosine-phosphorylated proteins were not visualized well in all experiments. As in the GST85 experiments, the ~180- and 130-kDa bands were not seen in anti-p85 immunoprecipitates of EGF-stimulated PC12 cells (Fig. 6), consistent with the lack of EGF-stimulated PtdIns 3-kinase activity in anti-ErbB3 immunoprecipitates from these cells (Fig. 1).

Tyrosine-phosphorylated proteins are immunoprecipitated by anti-ErbB3 antibody. To further demonstrate that ErbB3 is tyrosine phosphorylated in response to EGF, anti-ErbB3 immunoprecipitates of A431 cells were blotted with anti-P-Tyr antibody (Fig. 7). Tyrosine phosphorylation of several proteins was increased in anti-ErbB3 (K1) immunoprecipitates from EGF-treated A431 cells. These included a doublet at ~85 kDa, a broad band at ~95 kDa, a band at ~110 kDa, and two bands in the region of tyrosine kinase receptors. By using the 61-3 anti-ErbB3 antibody, increased tyrosine phosphorylation of the higher-molecular-mass band (~180 kDa) was also observed in EGF-treated A431 cells (not shown). Thus, the two anti-ErbB3 antibodies that immunoprecipitate EGF-stimulated PtdIns 3-kinase from A431 cells also immunoprecipitate a tyrosine-phosphorylated protein at ~180 kDa, the size at which ErbB3 migrates.

DISCUSSION

The results presented in this paper demonstrate the following: (i) EGF treatment of A431, PC12, and A549 cells causes the appearance of PtdIns 3-kinase activity in anti-P-Tyr immunoprecipitates, (ii) EGF-stimulated PtdIns 3-kinase activity is not found in anti-EGFR immunoprecipitates of the three cell lines by using several anti-EGFR antibodies, (iii) EGF-stimulated PtdIns 3-kinase activity is found in anti-ErbB3 immunoprecipitates of A431 cells but not PC12 or A549 cells, (iv) ErbB3 associates with anti-p85 antibody and recombinant p85 in EGF-treated A431 cells but not PC12 or A549 cells, and (v) ErbB3 is phosphorylated on tyrosine in EGF-treated A431 cells. These results suggest that ErbB3 mediates the EGF-promoted activation of PtdIns 3-kinase in A431 cells but not in PC12 cells or A549 cells. In addition to implicating ErbB3 in early signal transduction events initiated by EGF, these results suggest that there are alternative mechanisms by which EGF

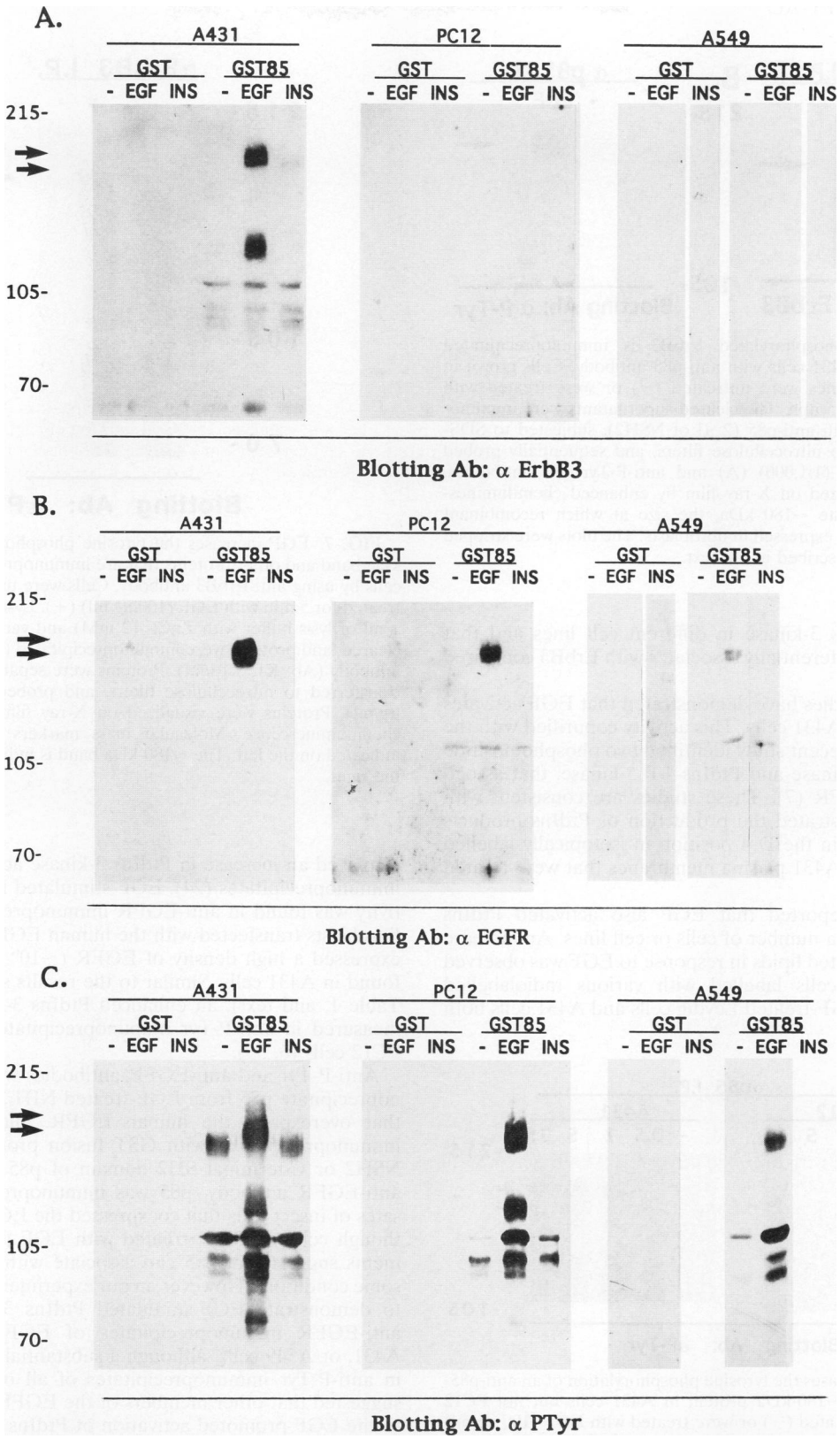

FIG. 4. GST85 precipitates ErbB3 from EGF-treated A431 cells but not from PC12 or A549 cells. Cells were untreated (−) or were treated for 5 min with EGF (100 ng/ml) or insulin (INS; 100 nM). The cleared supernatants were probed with GST or GST85, subjected to SDS-PAGE, and transferred to nitrocellulose filters. Molecular mass markers are indicated in kilodaltons on the left. The upper arrow indicates ~180 kDa, the size at which recombinant ErbB3 migrates when expressed in fibroblasts, and the lower arrow indicates ~170 kDa, the size of the EGFR. Proteins were visualized on X-ray film by enhanced chemiluminescence. (A) Filters were probed with anti-ErbB3 (49-3; 0.8 μg/ml) at 4°C overnight; (B) filters were stripped and reprobed with anti-EGFR (291-3A, 1:400 dilution) at 4°C overnight; (C) filters were stripped and reprobed with anti-P-Tyr antibody (1 μg/ml) overnight at 4°C.

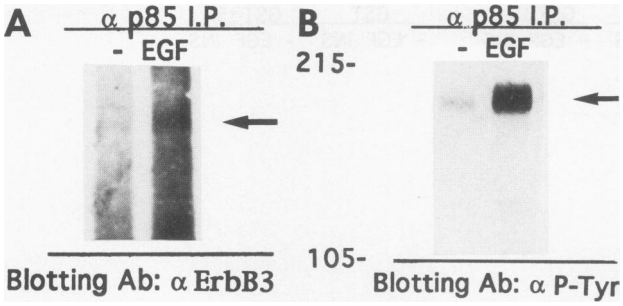

FIG. 5. Tyrosine-phosphorylated ErbB3 is immunoprecipitated from EGF-treated A431 cells with anti-p85 antibody. Cells grown in 100-mm-diameter dishes were untreated (−) or were treated with EGF (100 ng/ml) for 5 min. The cleared supernatants were immunoprecipitated (I.P.) with anti-p85 (2 μl of NSH2), subjected to SDS-PAGE, transferred to nitrocellulose filters, and sequentially probed overnight with 3183 (1:1,000) (A) and anti-P-Tyr (B) antibodies. Proteins were visualized on X-ray film by enhanced chemiluminescence. Arrows indicate ~180 kDa, the size at which recombinant ErbB3 migrates when expressed in fibroblasts. The blots were stripped between probes as described in the text.

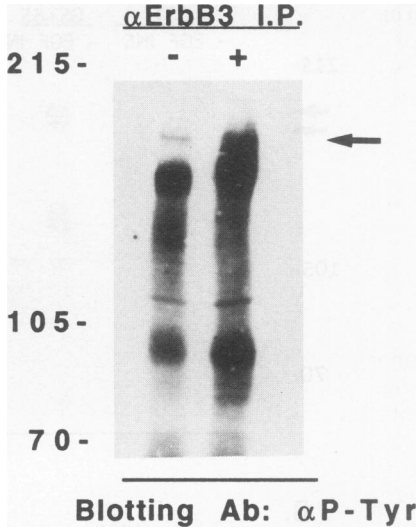

FIG. 7. EGF increases the tyrosine phosphorylation of an ~180-kDa band and other proteins that are immunoprecipitated from A431 cells by using anti-ErbB3 antibody. Cells were untreated (−) or were treated for 5 min with EGF (100 ng/ml) (+). Lysates were prepared in 1 ml of lysis buffer with $ZnCl_2$ (2 mM) and vanadate (1.2 mM) and cleared, and proteins were immunoprecipitated (I.P.) with anti-ErbB3 antibody (Ab; K1, 5 μl/ml). Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with anti-P-Tyr (1 μg/ml). Proteins were visualized on X-ray film by using enhanced chemiluminescence. Molecular mass markers (in kilodaltons) are indicated on the left. The ~180-kDa band is indicated by an arrow on the right.

can activate PtdIns 3-kinase in different cell lines and that PtdIns 3-kinase preferentially associates with ErbB3 compared with the EGFR.

A number of studies have demonstrated that EGF activates a PtdIns kinase in A431 cells. This activity copurified with the EGFR (42) and a recent study identified two phosphoinositide kinases, PtdIns 4-kinase and PtdIns-4-P 5-kinase, that associated with the EGFR (7). These studies are consistent with others that demonstrated the production of PtdIns products that were labelled in the D-4 position in isotopically labelled A431 cells (31) or A431 plasma membranes that were treated with EGF (27).

Other studies reported that EGF also activated PtdIns 3-kinase activity in a number of cells or cell lines. An increase in D-3-phosphorylated lipids in response to EGF was observed in Leydig tumor cells labelled with various radiolabelled precursors (30). EGF-treated Leydig cells and A431 cells both displayed an increase in PtdIns 3-kinase activity in anti-P-Tyr immunoprecipitates (24). EGF-stimulated PtdIns 3-kinase activity was found in anti-EGFR immunoprecipitates of mouse fibroblasts transfected with the human EGFR (2). These cells expressed a high density of EGFR (~10^6 per cell), like that found in A431 cells. Similar to the results shown here (Fig. 1, Table 1, and text), an enhanced PtdIns 3-kinase activity was measured in anti-P-Tyr immunoprecipitates of EGF-treated PC12 cells (6, 35).

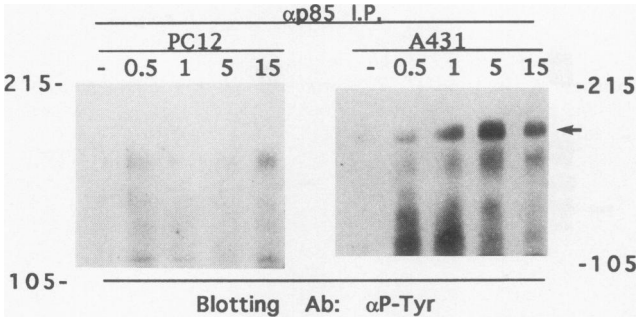

FIG. 6. EGF increases the tyrosine phosphorylation of an anti-p85-immunoprecipitable ~180-kDa protein in A431 cells but not PC12 cells. Cells were untreated (−) or were treated with EGF (100 ng/ml) for 0.5 to 15 min as indicated above each lane. The cleared supernatants were immunoprecipitated (I.P.) with anti-p85 (2 μl of NSH2), subjected to SDS-PAGE, transferred to nitrocellulose filters, and probed with anti-P-Tyr antibody (1 μg/ml) overnight at 4°C. Molecular mass markers are indicated to the left and right in kilodaltons. The arrow on the right indicates the ~180-kDa protein, the size at which recombinant ErbB3 migrates when expressed in fibroblasts. Proteins were visualized on X-ray film by enhanced chemiluminescence.

Anti-P-Tyr and anti-EGFR antibodies were used to immunoprecipitate p85 from EGF-treated NIH 3T3 (HER14) cells that overexpress the human EGFR, and the EGFR was immunoprecipitated with GST fusion proteins of either the NSH2 or C-terminal SH2 domain of p85 (14). By using an anti-EGFR antibody, p85 was immunoprecipitated from lysates of insect cells that coexpressed the EGFR and p85, even though cells were not treated with EGF (10). These experiments suggest that p85 can associate with the EGFR under some conditions. However, in our experiments, we were unable to demonstrate EGF-stimulated PtdIns 3-kinase activity in anti-EGFR immunoprecipitates of EGF-stimulated PC12, A431, or A549 cells, although a substantial activity was found in anti-P-Tyr immunoprecipitates of all of these cells. This suggested that other members of the EGFR family contribute to the EGF-promoted activation of PtdIns 3-kinase activity.

We investigated the possibility that the ErbB2 or Neu protein ($p185^{erbB2/neu}$) mediated the activation of PtdIns 3-kinase in response to EGF. In NIH 3T3 cells that expressed a chimera of the extracellular domain of the EGFR and the transmembrane and cytoplasmic domain of Neu, other investigators found that EGF-stimulated PtdIns 3-kinase activity was immunoprecipitated with anti-EGFR or anti-P-Tyr anti-

bodies (28). This suggests that the ErbB2 protein contains a binding site for PtdIns 3-kinase. Other studies have suggested that the EGFR and ErbB2 proteins interact and that ligands acting through the EGFR phosphorylate ErbB2 (16, 18). However, we did not detect PtdIns 3-kinase activity in anti-ErbB2 immunoprecipitates from EGF-stimulated A431, PC12, or A549 cells.

We detected ErbB3 in A431 cells that were probed with GST85. In these experiments, we identified proteins to which the p85 subunit could bind, and a protein identified by using an anti-ErbB3 antibody was found at ~180 kDa in EGF-treated A431 cells but not in PC12 or A549 cells (Fig. 4A). Tyrosine phosphorylation of a protein at ~180 kDa was also observed in anti-p85 immunoprecipitates (Fig. 6) and anti-ErbB3 immunoprecipitates (Fig. 7) of EGF-stimulated A431 cells. This protein was identified as ErbB3 in the anti-p85 immunoprecipitates. Consistent with these results, expression of the human *erbB3* gene in NIH 3T3 fibroblasts resulted in the production of a 180-kDa glycoprotein (19). This protein was constitutively tyrosine phosphorylated, and EGF stimulated the tyrosine phosphorylation of a chimera of the extracellular domain of the EGFR and the intracellular domain of ErbB3 expressed in NIH 3T3 cells (19). In a paper that was published while the manuscript of this paper was being revised, EGF was found to stimulate the association of PtdIns 3-kinase with a chimeric EGFR/ErbB3 receptor consisting of the extracellular and transmembrane domain of EGFR with the cytosolic domain of ErbB3 (9). While that report and our data demonstrate the association of PtdIns 3-kinase with the cytosolic domain of ErbB3, suggesting that ErbB3 can serve to recruit PtdIns 3-kinase to the plasma membrane of A431 cells, our results additionally suggest that the EGFR is involved in this process under in vivo physiological conditions. In this model, EGF activates the intrinsic tyrosine kinase activity of the EGFR, resulting in the tyrosine phosphorylation of ErbB3 on the appropriate residue(s) to which PtdIns 3-kinase is recruited. As mentioned in the Introduction, ErbB3 has seven Tyr-X-X-Met sites that are potential PtdIns 3-kinase binding motifs. ErbB3 is expressed in other human mammary tumor cell lines (20, 33), and our current studies are investigating its role in the activation of these cells by EGF and the recruitment of other signalling proteins to ErbB3.

These results suggest that the relationship between ErbB3 and the EGFR is analogous to that between IRS-1 and the insulin receptor or insulin-like growth factor I receptor. Insulin promotes the tyrosine phosphorylation of the β subunit of the insulin receptor (reviewed in reference 36). In response to insulin, IRS-1 associates with the insulin receptor, and IRS-1 itself becomes phosphorylated on tyrosine (40). Subsequently, PtdIns 3-kinase is recruited to IRS-1 (17, 40). There are nine potential p85 binding sites on rat IRS-1 (39). In insulin-treated cells, PtdIns 3-kinase activity is greatly enhanced in anti-IRS-1 immunoprecipitates or anti-P-Tyr immunoprecipitates (40, 41), but much less stimulation is observed in anti-insulin receptor immunoprecipitates (8, 37). Moreover, it has been difficult to detect the insulin receptor in anti-p85 immunoprecipitates of insulin-treated cells (11), although the insulin receptor can be detected in anti-IRS-1 immunoprecipitates of cells that overexpressed IRS-1 (41). Thus, IRS-1 functions as an accessory protein to the insulin receptor for the recruitment of PtdIns 3-kinase and activation of PtdIns 3-kinase activity. We suggest that ErbB3 serves the same function for the EGFR. One difference between these two proteins is that IRS-1 is a cytosolic protein, while ErbB3 is a transmembrane protein. However, CD19, a membrane protein found in B lymphocytes, also has a Tyr-X-X-Met motif, and PtdIns 3-kinase bound to CD19 that was cross-linked to membrane immunoglobulin M (43).

In conclusion, our results indicate that ErbB3 is involved in the activation of PtdIns 3-kinase activity by EGF in A431 cells but not in two other cell lines. In addition to implicating the ErbB3 protein in early events in EGF-initiated signal transduction, it appears that the EGF-promoted signal transduction pathway involved in mitogenic responses is different in different types of cells. Presently, since there are four members of the type I receptor kinase family (EGFR, ErbB2, ErbB3, and ErbB4), the possibility remains that ErbB3 associates with other members of this family and serves as the PtdIns 3-kinase binding protein. Levels of the EGFR are much higher in A431 cells than in PC12 or A549 cells. The involvement of ErbB3 in the EGF-stimulated activation of PtdIns 3-kinase may be a consequence of the high receptor number. If so, this may play a role in tumor formation or progression, processes that are associated with the overexpression of members of the class I receptor family.

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