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Efficient Coupling with Phosphatidylinositol 3-Kinase, but Not Phospholipase Cγ or GTPase-Activating Protein, Distinguishes ErbB-3 Signaling from That of Other ErbB/EGFR Family Members

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Recombinant expression of a chimeric EGFR/ErbB-3 receptor in NIH 3T3 fibroblasts allowed us to investigate cytoplasmic events associated with ErbB-3 signal transduction upon ligand activation. An EGFR/ErbB-3 chimera was expressed on the surface of NIH 3T3 transfectants as two classes of receptors possessing epidermal growth factor (EGF) binding affinities comparable to those of the wild-type EGF receptor (EGFR). EGF induced autophosphorylation in vivo of the chimeric receptor and DNA synthesis of EGFR/ErbB-3 transfectants with a dose response similar to that of EGFR transfectants. However, the ErbB-3 and EGFR cytoplasmic domains exhibited striking differences in their interactions with several known tyrosine kinase substrates. We demonstrated strong association of phosphatidylinositol 3-kinase activity with the chimeric receptor upon ligand activation comparable in efficiency with that of the platelet-derived growth factor receptor, while the EGFR exhibited a 10- to 20-fold-lower efficiency in phosphatidylinositol 3-kinase recruitment. By contrast, both phospholipase Cγ and GTPase-activating protein failed to associate with or be phosphorylated by the ErbB-3 cytoplasmic domain under conditions in which they coupled with the EGFR. In addition, though certain signal transmitters, including Shc and GRB2, were recruited by both kinases, EGFR and ErbB-3 elicited tyrosine phosphorylation of distinct sets of intracellular substrates. Thus, our findings show that ligand activation of the ErbB-3 kinase triggers a cytoplasmic signaling pathway that hitherto is unique within this receptor subfamily.

Signal transduction by receptor tyrosine kinases (RTKs) involves tyrosine phosphorylation and/or physical association of a number of cytosolic enzymes by the activated receptor. Initially identified as immediate targets of the platelet-derived growth factor (PDGF) receptor (PDGFR) and the epidermal growth factor (EGF) receptor (EGFR), these substrates include phospholipase Cγ (PLCγ), GTPase-activating protein (GAP), and phosphatidylinositol 3-kinase (PtdIns 3-kinase) (2, 11, 13, 24, 36, 39, 41, 58, 64). Their coding sequences share regions of homology with the noncatalytic portion of c-*src*, termed SH2 and SH3, that are involved in the assembly of signaling complexes with the activated receptor. Thereby, SH2 domains bind to discrete phosphotyrosine (P-Tyr)-containing peptide sequences present in RTKs, whereas SH3 domains appear to associate with proline-rich motifs in proteins regulating the activity of low-molecular-mass G proteins (5, 14, 26, 43, 49, 52, 69).

Although biochemical functions of these substrates are well characterized, their biological relevance for mitogenic signaling has not unequivocally been established. For instance, PLCγ activation by RTKs correlates with increased phosphatidylinositol bisphosphate hydrolysis and cell proliferation (35, 66). Furthermore, microinjection of purified PLCγ has been reported to cause DNA synthesis and morphological transformation of NIH 3T3 cells (59). However, recombinant PLCγ overexpression did not enhance the mitogenic response to PDGF, despite increased phosphatidylinositol bisphosphate turnover (37). Likewise, mutational analysis of RTKs indicated that PLCγ might be dispensable for mitogenesis (40, 46). Similarly, although PtdIns 3-kinase activity has been linked to the proliferative action of the v-Src protein (17), abrogation of PtdIns 3-kinase association with the αPDGFR did not affect mitogenic or chemotactic signals (68). These observations might be reconciled by a possible redundance of intracellular signaling pathways. In fact, a recent study indicated that among PLCγ, PtIns 3-kinase, GAP, and a 64-kDa substrate protein, either PLCγ or PtIns 3-kinase is sufficient to transduce a PDGF mitogenic signal (62). Whatever the case, discrete patterns of substrate interaction by different receptors as well as distinct biological activities of the same receptor when expressed in different cell types have implied that the biological response to a growth factor is modulated by the ability of a receptor to activate specific signaling cascades (3, 54).

We are interested in the characterization of the biochemical and biological properties of the *erb*B-3 gene product. The *erb*B-3 gene has been isolated as a third member of the ErbB/EGFR family (RTK type I) on the basis of close structural similarity with EGFR and *erb*B-2 (28, 47, 61). Its gene product has been characterized as a transmembrane glycoprotein, gp180$^{erbB\text{-}3}$, possessing functional properties of a growth factor receptor-like tyrosine kinase (27). Chronic tyrosine phosphorylation of gp180$^{erbB\text{-}3}$ in certain mammary tumor cell lines implicated constitutive activation of the ErbB-3 tyrosine kinase in human neoplasia (27). Under physiological conditions, ErbB-3 is expressed in cells of epithelial and neuroectodermal derivation, thus suggesting a

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role of ErbB-3 signaling in these tissues (28). Frequent coexpression with the EGFR and ErbB-2 raises the possibility that ErbB-3 signaling specificity is determined by qualitative differences in the recruitment of second messengers. To date, a natural ligand regulating the ErbB-3 catalytic activity has not been described. In efforts to study cytoplasmic events involved in ErbB-3-mediated signal transduction, we engineered a chimeric EGFR/ErbB-3 receptor containing the ligand-binding domain of the EGFR and the cytoplasmic domain of ErbB-3. A similar approach has previously been used for the analysis of the signaling pathway of other RTKs with unknown ligand activities (20, 30, 31, 33, 50, 55, 67). Comparing EGF-dependent signal transduction in LTR (long terminal repeat)-EGFR/ErbB-3 and LTR-EGFR transfectants, we demonstrate in this study qualitative and quantitative differences in the ability of these two kinases to couple with intracellular signaling pathways.

MATERIALS AND METHODS

Generation of expression vectors and transfection. The chimeric EGFR/ErbB-3 receptor was engineered by joining extracellular and transmembrane domains of the EGFR (amino acids 1 to 682, corresponding to positions −24 to 658 in reference 60) with the intracellular portion of ErbB-3 (amino acids 681 to 1342 [28]). For its construction, we used an EGFR cDNA containing a unique *Sal*I site in the juxtatransmembrane domain of the coding sequence (33). To facilitate recombination, an identical restriction site was generated in the homologous region of the *erb*B-3 coding sequence (position 2135), using recombinant PCR for site-directed mutagenesis on a unique *Dra*III-*Bam*HI *erb*B-3 segment (nucleotide positions 1856 to 2284 [28]). The 2.1-kbp *Xho*I-*Sal*I fragment of the EGFR and the 2.1-kbp *Sal*I fragment of intracellular *erb*B-3 coding sequence were gel purified and ligated in the unique *Sal*I cloning site of $ZIPneo_{Sal}$ (27) to obtain the LTR-EGFR/ErbB-3 expression vector. Cloning boundaries and PCR-amplified regions were confirmed by nucleotide sequence analysis to verify sense orientation and integrity of the open reading frame. The linearized expression construct (0.01 to 10 μg per plate) was transfected into NIH 3T3 cells by calcium phosphate precipitation, using 40 μg of calf thymus DNA as the carrier. Mass cultures expressing the recombinant protein were obtained by selection with 750 μg of G418 per ml. Selected LTR-EGFR/ErbB-3 transfectants were enriched for expression of the chimeric protein by preparative fluorescence-activated cell sorting, using EGFR monoclonal antibody AB-1 (Oncogene Sciences). NIH 3T3 cells transfected with $ZIPneo_{Sal}$ and marker selected served as the negative control (LTR-neo). The LTR-EGFR transfectant has been previously described (8).

EGF binding and mitogenic assays. For EGF binding and mitogenic assays, 10^5 cells were seeded in triplicate on fibronectin-coated 24-well plates. Specific EGF binding was determined following subtraction of nonspecific binding, using [^{125}I]EGF (Amersham) at concentrations ranging from 0.01 to 300 ng/ml. EGF binding sites and dissociation constants were extrapolated by Scatchard analysis essentially as described previously (8). DNA synthesis was measured by [^{3}H]thymidine incorporation as reported previously (8) and was expressed as fold increase of [^{3}H]thymidine incorporation at the indicated EGF concentrations above background determined in serum-free medium.

Protein analysis. Immunoprecipitation and immunoblot analysis was conducted as previously described (27). For immunoprecipitations with monoclonal anti-EGFR (AB-1; Oncogene Sciences) and anti-P-Tyr PY20 and PY69; ICN) antibodies, Gammabind G agarose was precoated with goat anti-mouse immunoglobulin G secondary antibody (Boehringer Mannheim). For coimmunoprecipitation experiments, lysates were freshly prepared in Nonidet P-40 (NP-40) buffer (20 mM Tris-HCl [pH 7.6], 100 mM NaCl, 5 mM EDTA, 1% NP-40, 10 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4). Immunoprecipitates were washed in phosphate-buffered saline (PBS) containing 1% NP-40 and 2 mM Na_3VO_4. Antibodies directed against PLCγ and p85 were purchased from Upstate Biotechnologies Inc. Peptide antisera specific for the EGFR (6), ErbB-3 (27), or GAP (42, 48) have been previously described. Coimmunoprecipitation experiments with a polyclonal anti-Shc antibody (catalog no. S14630; Transduction Laboratories) were conducted on fresh lysates prepared in 1% Triton buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 150 mM NaCl, 1% Triton, 5 mM EGTA) containing protease and phosphatase inhibitors. GRB2 was detected in immunoblot analysis using the anti-GRB2 monoclonal antibody GR81 (Transduction Laboratories) and a secondary anti-mouse immunoglobulin antibody.

PtdIns 3-kinase assay. For measuring receptor-associated PtdIns 3-kinase activity in vivo, quiescent transfectants were mock treated or exposed for 10 min to 100 ng EGF or PDGF per ml at 37°C and instantly lysed in NP-40 lysis buffer. Following three washes in PBS–1% NP-40–2 mM Na_3VO_4 and two washes in 0.1 M Tris-Cl (pH 7.6)–0.5 M LiCl, immunoprecipitates were equilibrated in 20 mM Tris-Cl (pH 7.6)–100 mM NaCl–5 mM EDTA and finally in 20 mM HEPES (pH 7.6). Immunoprecipitates were resuspended in 50 μl of reaction buffer (20 mM Tris-Cl [pH 7.6], 100 mM NaCl, 0.5 mM EGTA) and monitored for PtdIns 3-kinase activity by its ability to phosphorylate phosphatidylinositol (Sigma) in the presence of [γ-^{32}P]γATP (1 μCi) and $MgCl_2$ (5 mM). The reaction proceeded for 15 min at 25°C and was stopped by addition of 100 μl of 1 M HCl. Following chloroform extraction, the phosphorylated product was subjected to thin-layer chromatography in a buffer containing 22 volumes of chloroform, 19 volumes of methanol, 4 volumes of H_2O, and 1 volume of ammonium hydroxide. Reaction products were visualized by autoradiography and identified by comparison with phospholipid standards.

RESULTS

An EGFR/ErbB-3 chimera transduces EGF-dependent signals in fibroblasts. The LTR-EGFR/ErbB-3 expression vector (Fig. 1A) was engineered by joining extracellular and transmembrane domains of the EGFR (amino acids 1 to 682) with the intracellular portion of ErbB-3 (amino acids 681 to 1342) and was transfected into NIH 3T3 cells. Selected LTR-EGFR/ErbB-3 mass cultures expressed a 180-kDa chimeric protein that was detected by antibodies specific for the extracellular domain of the EGFR and the cytoplasmic domain of ErbB-3 (27) (Fig. 2). EGF-dependent tyrosine phosphorylation of the cytoplasmic ErbB-3 domain demonstrated its ability to transduce EGF signals intracellularly (27) (Fig. 2). To quantitate chimeric receptor numbers, selected mass cultures were analyzed for EGF binding in comparison with NIH 3T3 cells overexpressing the EGFR (LTR-EGFR [8]). Scatchard analysis of [^{125}I]EGF binding kinetics established that the two cell lines expressed comparable amounts of EGF binding sites in the order of 10^6 per cell (Table 1). In NIH 3T3 transfectants, both EGFR and

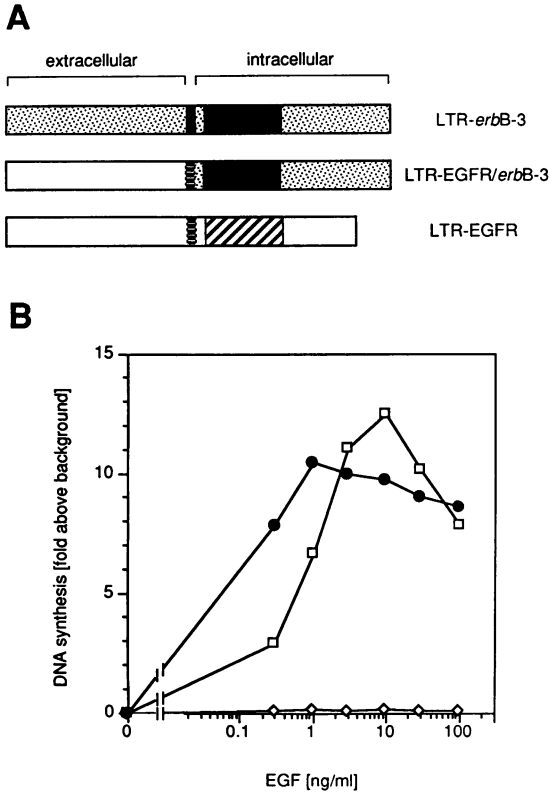

FIG. 1. EGF signal transduction by an EGFR/ErbB-3 chimera. (A) In the chimeric receptor, predicted ligand-binding and transmembrane domains of ErbB-3 have been replaced by the homologous regions of the EGFR. (B) EGF-dependent DNA synthesis of LTR-EGFR/ErbB-3 (●), LTR-EGFR (□), and LTR-neo (◆) transfectants expressing 1×10^6, 3×10^6, and $<3 \times 10^3$ EGF binding sites per cell, respectively.

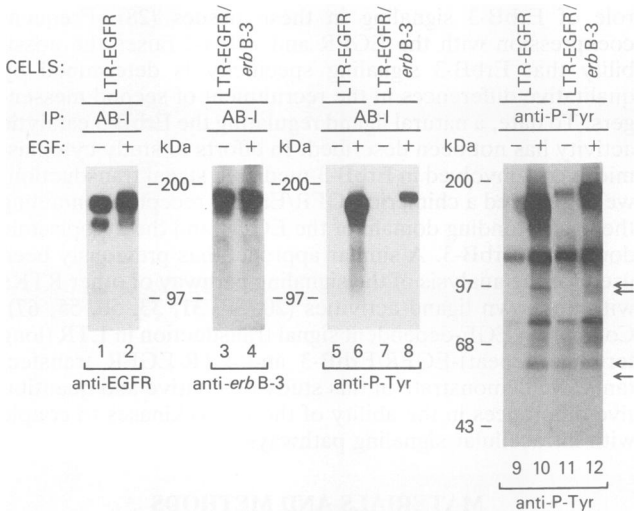

FIG. 2. Tyrosine phosphorylation of cellular proteins induced by the ErbB-3 or EGFR kinase. Lysates (2 mg) of mock-treated or EGF-triggered transfectants were immunoprecipitated (IP) with a monoclonal antibody reactive with the EGFR extracellular domain and subjected to immunoblot analysis with receptor-specific peptide antisera (lanes 1 to 4) or anti-P-Tyr antibodies (lanes 5 to 8). Cellular P-Tyr proteins were detected by sequential immunoprecipitation from 4 mg of total cellular protein and immunoblotting with anti-P-Tyr antibodies (lanes 9 to 12).

EGFR/ErbB-3 were expressed as two classes of receptors, displaying high (~0.1 nM) and low (>4 nM) affinities for EGF binding. High-affinity sites represented less than 10% of the total receptor pool. By comparison, LTR-neo control transfectants expressed less than 3×10^3 endogenous EGF binding sites.

We next examined whether the ErbB-3 intracellular domain was capable of transducing a mitogenic signal. When the LTR-EGFR/ErbB-3 transfectant was exposed to increasing EGF concentrations, there was a dose-dependent stimulation of DNA synthesis similar to that observed with LTR-EGFR transfectants (Fig. 1B). By comparison, the LTR-neo transfectant showed no significant increase of DNA synthesis over the range of EGF concentrations tested, indicating that low-level endogenous EGFR in NIH 3T3 cells did not yield a mitogenic EGF response under these conditions (Fig. 1B). Thus, the EGFR/ErbB-3 chimeric receptor was correctly processed and expressed at the cell surface, where it was capable of binding EGF and transducing a mitogenic signal.

The ErbB-3 and EGFR kinases induce tyrosine phosphorylation of distinct subsets of cellular proteins. We compared the pattern of P-Tyr-containing proteins in LTR-EGFR/ErbB-3 and LTR-EGFR transfectants by consecutive immunoprecipitation and immunoblotting using anti-P-Tyr antibodies (PY20 and PY69; ICN). As shown in Fig. 2, lysates from EGF-triggered LTR-EGFR/ErbB-3 transfectants contained a prominent 180-kDa P-Tyr-containing protein that represented the autophosphorylated chimeric receptor (Fig. 2, lane 4, 8, and 12). In addition, tyrosine phosphorylation of bands with apparent molecular masses of 170, 125, 97, 95, 80, and 70 kDa were reproducibly induced by EGF in chimeric receptor transfectants (Fig. 2, lane 12) but not LTR-neo control cells (data not shown). In immunoblot analysis of LTR-EGFR/ErbB-3 cells, carboxyl-terminal ErbB-3 peptide antisera specifically recognized a 180-kDa and a 170-kDa species (data not shown), suggesting that the latter represented another phosphorylated form of the chimeric receptor. Under these conditions, none of the smaller-molecular-mass proteins phosphorylated in EGF-triggered chimeric receptor transfectants was detectable, suggesting that the bands of 125, 97, 95, 80, and 70 kDa represented ErbB-3 substrates rather than receptor degradation products.

Comparison of cellular tyrosine phosphorylation patterns induced by the ErbB-3 and EGFR kinases (Fig. 2, lane 10 and 12) indicated differences in substrate phosphorylation. In particular, a doublet of 97- and 95-kDa proteins that were tyrosine phosphorylated in LTR-EGFR/ErbB-3 cells ap-

TABLE 1. [^{125}I]EGF binding properties of EGFR/ErbB-3 and EGFR transfectants[a]

NIH 3T3 transfectant	High affinity		Low affinity	
	Receptors/cell	K_d (nM)	Receptors/cell	K_d (nM)
EGFR/ErbB-3	8×10^4	0.1	1×10^6	4.2
EGFR	2×10^5	0.1	3×10^6	5.5

[a] [^{125}I]EGF binding was assessed over a range of concentrations from 0.016 to 50 nM in triplicate wells. Specificity of binding was controlled by competition with a 100-fold excess of unlabeled EGF. Data are derived from Scatchard plot transformations of binding values.

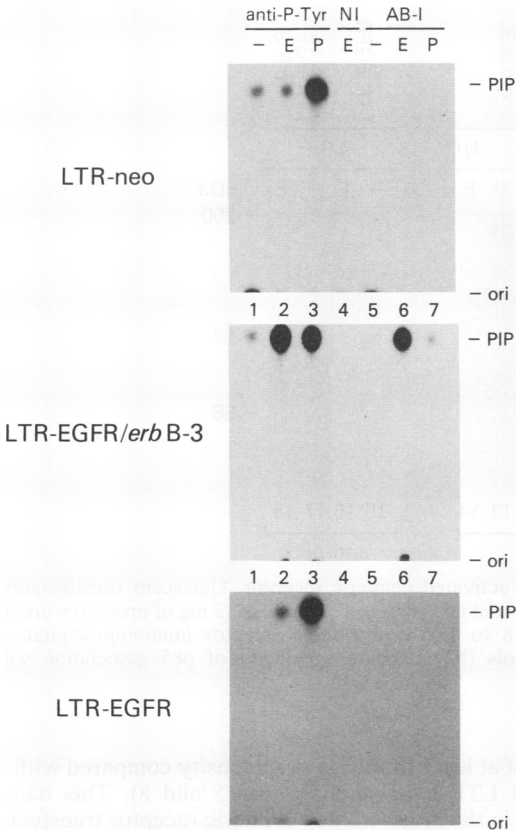

FIG. 3. EGF-dependent association of PtdIns 3-kinase activity with an EGFR/ErbB-3 chimera. Lysates (2 mg) of mock-treated (–), EGF (E)-triggered, or PDGF (P)-triggered transfectants immunoprecipitated with anti-P-Tyr or a monoclonal antibody directed against the EGFR extracellular domain (AB-1) as well as a nonimmune control (NI). Immunoprecipitates were assayed for PtdIns 3-kinase activity as described in Materials and Methods. The reaction products were subjected to thin-layer chromatography. Origin (ori) and migration position of phosphatidylinositol 3-phosphate (PIP) are indicated.

peared to be distinct from a single band of slightly larger molecular mass which was phosphorylated upon activation of the EGFR. Furthermore, two bands of 60 and 63 kDa induced in EGFR transfectants did not appear to be ErbB-3 substrates. In contrast, bands of 125 and 80 kDa showed increased tyrosine phosphorylation in both LTR-EGFR/ErbB-3 and LTR-EGFR transfectants, suggesting that those may represent substrate proteins shared by ErbB-3 and EGFR kinases. Thus, comparison of tyrosine phosphorylation patterns in LTR-EGFR/ErbB-3 and LTR-EGFR transfectants upon EGF triggering suggested that ErbB-3 and EGFR signaling pathways in fibroblasts are at least in part distinct.

Efficient recruitment of PtdIns 3-kinase activity by ligand-activated EGFR/ErbB-3. In an attempt to delineate differences in ErbB-3 and EGFR signal transduction, we compared the abilities of the two kinases to couple with known biochemical pathways implicated in mitogenic signaling. One such pathway involves recruitment of the PtdIns 3-kinase complex by certain RTKs upon ligand stimulation, resulting in association of PtdIns 3-kinase activity with the fraction of P-Tyr proteins (22, 24, 53, 63). As shown in Fig. 3, EGF triggering induced a pronounced increase in the recovery of PtdIns 3-kinase activity from anti-P-Tyr immunoprecipitates of LTR-EGFR/ErbB-3 transfectants but not LTR-neo cells. This increase in PtdIns 3-kinase activity was slightly higher than that observed following activation of the PDGFR in the same cells and at least 10-fold greater than that recovered from EGF-triggered LTR-EGFR cells under comparable conditions (Fig. 3, lanes 1 to 3).

To determine whether PtdIns 3-kinase activity directly associated with the chimeric receptor, we immunoprecipitated identical lysates with a monoclonal antibody specific for the EGFR extracellular domain. PtdIns 3-kinase activity was readily detected in immunoprecipitates of EGF-triggered LTR-EGFR/ErbB-3 transfectants and at significantly lower levels in EGF-stimulated LTR-EGFR cells (Fig. 3, lanes 5 to 7). An approximately twofold-higher background signal following PDGF triggering of LTR-EGFR/ErbB-3 transfectants compared with mock-treated controls was not reproducibly observed. Specificity of coimmunoprecipitation was confirmed by lack of PtdIns 3-kinase activity in parallel immunoprecipitations using nonimmune serum (Fig. 3, lane 4). All of these findings established that an active ErbB-3 kinase recruits PtdIns 3-kinase activity with an efficiency comparable to that of the activated PDGFR and at least 10-fold more efficiently than the EGFR.

Association of the p85 subunit of PtdIns 3-kinase with activated EGFR/ErbB-3 receptor. Purified PtdIns 3-kinase has been characterized as a heterodimer consisting of a p85 and a p110 subunit (4, 21). The latter has been shown to contain the enzymatic activity, while p85 has been implicated in the association of the enzyme with the phosphorylated receptor (2, 3, 12, 13, 21, 58). As an independent measure for PtdIns 3-kinase recruitment, we investigated whether p85 complexes with the ErbB-3 cytoplasmic domain in LTR-EGFR/ErbB-3 transfectants. As shown in Fig. 4 (lanes 1 to 6), similar amounts of p85 were immunoprecipitated from untreated, PDGF-treated, and EGF-treated NIH 3T3 transfectants. Immunoblot analysis of the same immunoprecipitates with anti-P-Tyr antibodies revealed coimmunoprecipitation of tyrosine-phosphorylated proteins whose molecular weights corresponded with those of EGFR/ErbB-3, EGFR, or PDGFR (lanes 8 to 13). Coimmunoprecipitation of the activated chimeric receptor was dependent on the presence of p85 in the immunoprecipitate (lanes 7 and 14), demonstrating specificity of this association. At similar receptor expression levels, there was significantly less EGFR association with p85 upon EGF triggering of LTR-EGFR cells (lane 9). As a control, PDGF stimulation led to similar coimmunoprecipitation of activated PDGFRs in LTR-EGFR/ErbB-3 and LTR-EGFR cells (lanes 10 to 13).

These findings were paralleled by p85 coimmunoprecipitation in experiments using antireceptor antibodies. The amount of p85 associated with the chimeric receptor was substantially increased upon EGF triggering of LTR-EGFR/ErbB-3 cells. By comparison, significantly less p85 coimmunoprecipitated following EGF activation of EGFRs (Fig. 4, lanes 15 to 18). These observations demonstrated that upon ligand activation, the ErbB-3 cytoplasmic domain was more efficient than that of the EGFR in p85 recruitment.

EGF signal transduction by EGFR/ErbB-3 lacks significant activation of PLCγ and GAP pathways. EGFR-mediated signal transduction has previously been shown to induce tyrosine phosphorylation of PLCγ and GAP, although at reduced stoichiometry compared with PDGFR signaling in NIH 3T3 cells (3, 16). We therefore compared the efficiency of the ErbB-3 kinase with that of EGFR and PDGFR to activate these pathways. Immunoblot analysis of anti-P-Tyr

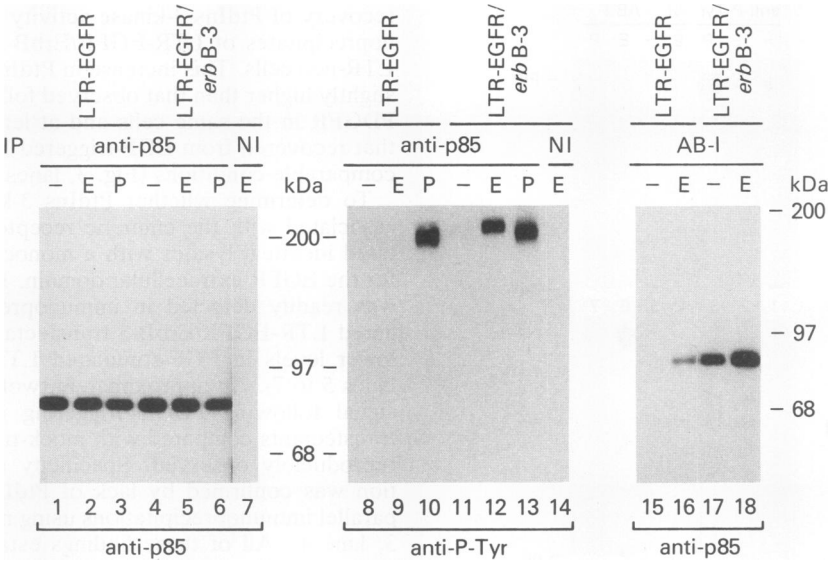

FIG. 4. Coimmunoprecipitation of p85 subunit of PtdIns 3-kinase with the activated chimeric receptor. Quiescent transfectants were exposed to EGF (E) or PDGF (P). Anti-p85 immunoprecipitates (IP) of mock-treated (−) or triggered samples (2 mg of protein) were divided for immunoblot analysis with anti-p85 (lanes 1 to 6) or anti-P-Tyr (lanes 8 to 13). Conversely, receptor immunoprecipitates were immunoblotted with anti-p85 antiserum (lane 15 to 18). Nonimmune controls (NI) indicate specificity of p85 association with the phosphorylated chimeric receptor.

immunoprecipitates with an anti-PLCγ monoclonal antibody (05-163; Upstate Biotechnologies Inc.) revealed similarly high levels of tyrosine-phosphorylated PLCγ upon PDGF triggering of LTR-neo, LTR-EGFR, and LTR-EGFR/ErbB-3 cells (Fig. 5, lanes 3, 6, and 9). By comparison, EGF treatment of LTR-EGFR cells induced the appearance of PLCγ in anti-P-Tyr immunoprecipitates at three- to fivefold-lower efficiency than PDGF stimulation (lanes 5 and 6). These findings were in accordance with the lower efficiency of the EGFR than of the PDGFR to induce tyrosine phosphorylation of PLCγ. By comparison, EGF triggering of LTR-EGFR/ErbB-3 transfectants revealed a faint PLCγ band of at least 10-fold-lower intensity compared with EGF-treated LTR-EGFR cells (lanes 5 and 8). This band was absent in the mock-treated chimeric receptor transfectant or EGF-stimulated LTR-neo cells (lanes 2 and 7), indicating that its appearance was dependent on activation of the chimeric receptor.

Immunoblot analysis of anti-PLCγ immunoprecipitates with anti-P-Tyr antibodies demonstrated coimmunoprecipitation of tyrosine-phosphorylated PLCγ and EGFR (Fig. 5, lane 20). We reproducibly observed a protein of 97 kDa coimmunoprecipitating with PLCγ upon EGF treatment of LTR-EGFR cells (lane 20); the identity of this protein,

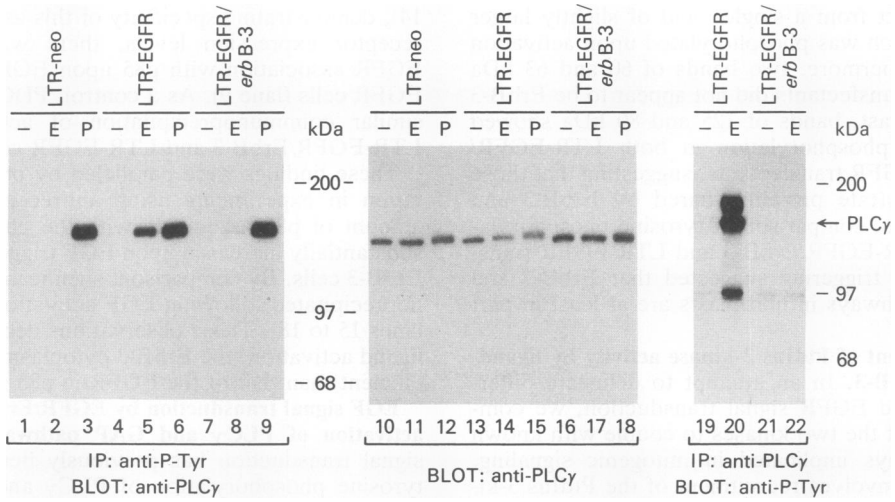

FIG. 5. Efficient PLCγ recruitment by PDGFR and EGFR but not the ErbB-3 cytoplasmic domain. Quiescent transfectants were treated for 10 min at 37°C as follows: −, mock; E, 100 ng of EGF per ml; P, 100 ng of PDGF per ml. Two milligrams of lysates was used in immunoprecipitations (IP) with anti-P-Tyr (lanes 1 to 9) or anti-PLCγ (lanes 10 to 18) antibodies, whereas direct immunoblot analysis of PLCγ (lanes 10 to 18) was conducted with 100 μg of total cellular protein.

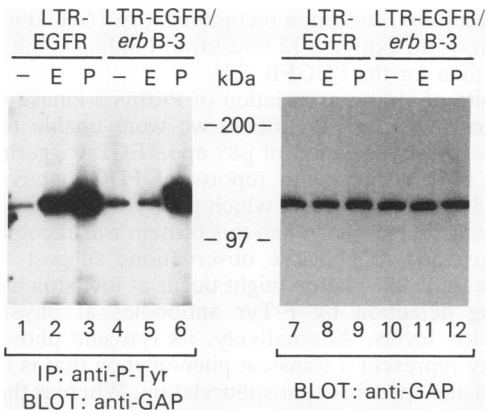

FIG. 6. Ligand activation of the ErbB-3 cytoplasmic domain does not involve GAP recruitment. Anti-GAP immunoblot analysis on anti-P-Tyr immunoprecipitates (IP) or total protein lysates was conducted as described for PLCγ analysis (see the legend to Fig. 5). The left panel represents a prolonged film exposure to visualize GAP recruitment by the EGFR.

however, is not known. Conversely, EGF triggering of the chimeric receptor did not induce coimmunoprecipitation of either phosphorylated PLCγ or the 97-kDa phosphoprotein (lane 22). These findings indicated that at comparable receptor expression levels, the ErbB-3 kinase is significantly less efficient than the EGFR kinase in activating the PLCγ signaling pathway.

Similar observations were obtained when we analyzed ErbB-3 interaction with GAP. Under conditions in which we readily detected GAP tyrosine phosphorylation by PDGFR or EGFR, there was no evidence for increased GAP tyrosine phosphorylation following EGF treatment of LTR-EGFR/ErbB-3 transfectants (Fig. 6). Therefore, in NIH 3T3 fibroblasts, neither PLCγ nor GAP appears to represent a major component of the ErbB-3 cytoplasmic signaling pathway.

EGF-dependent recruitment of Shc and GRB2 in EGFR/ErbB-3 transfectants. Shc and GRB2 have recently been implicated in the activation of the *ras* pathway by tyrosine kinases, including EGFR, PDGFR, and insulin receptor (1, 10, 18, 32, 34, 45, 51, 52, 57). We investigated whether ErbB-3 can couple with this signaling cascade. Immunoblot analysis using anti-P-Tyr antibodies of anti-Shc immunoprecipitates revealed EGF-dependent Shc tyrosine phosphorylation in LTR-EGFR/ErbB-3 significantly exceeding that of LTR-neo cells (Fig. 7A). Enhanced induction of Shc phosphorylation in LTR-EGFR transfectants compared with LTR-EGFR/ErbB-3 cells (Fig. 7A, lanes 4 and 6) corresponded to the relative levels of receptor tyrosine phosphorylation (Fig. 2, lanes 6 and 8) and was probably due to the different expression levels of EGFR and EGFR/ErbB-3 in these transfectants (Table 1). Reblotting of the same filter confirmed that similar amounts of the described Shc proteins p47, p52, and p66 were immunoprecipitated in all lanes (data not shown). Among these isoforms, tyrosine phosphorylation was most pronounced on p52 (Fig. 7A). These observations identified Shc as a substrate for the ErbB-3 kinase.

In EGF-triggered LTR-EGFR/ErbB-3 transfectants, coimmunoprecipitation of a 180-kDa P-Tyr-containing band comigrating with the chimeric receptor (Fig. 7A, lane 6) indicated that Shc associated with the ErbB-3 cytoplasmic domain of the activated EGFR/ErbB-3 receptor. This observation paralleled findings with LTR-EGFR controls in which a 170-kDa

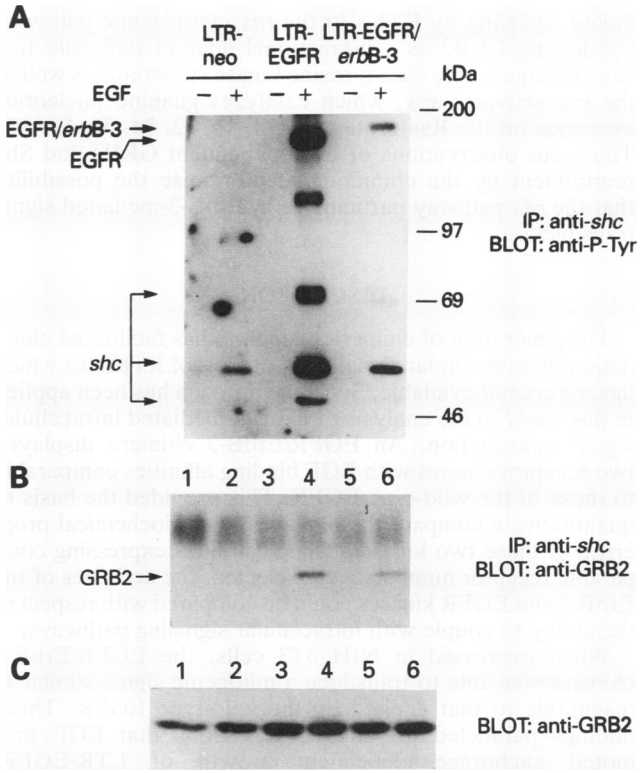

FIG. 7. EGF-dependent recruitment of Shc and GRB2 by the chimeric EGFR/ErbB-3 receptor. (A) For each lane, 1 mg of lysate was immunoprecipitated (IP) with an anti-Shc polyclonal antibody, separated on a 7.5% polyacrylamide gel, and subjected to immunoblot analysis with anti-P-Tyr antibodies. Retardation in electrophoretic mobility of p47, p52, and p66 is due to phosphorylation. (B) Anti-Shc immunoprecipitates from 1 mg of lysate were electrophoresed on a 12% polyacrylamide gel and immunoblotted with an anti-GRB2 antibody. The diffuse band present in all lanes and trailing the GRB2 protein represents light chains of immunoprecipitation antibody. (C) Aliquots of 100 μg of the same lysates as in panel B were subjected to direct immunoblot analysis with the anti-GRB2 antibody.

band corresponded to the phosphorylated EGFR (lane 4). The nature of an additional ~130-kDa tyrosine-phosphorylated protein in this lane is unknown. However, a P-Tyr-containing protein of similar molecular weight derived from lysates of EGF-triggered EGFR transfectants has previously been reported to associate with GRB2 and the activated EGFR in vitro (34). It is therefore conceivable that the 130-kDa phosphoprotein represents an as yet unidentified component of the EGFR signaling complex.

We subsequently investigated whether activation of the chimeric receptor resulted in GRB2 recruitment. Immunoblot analysis using an anti-GRB2 antibody demonstrated EGF-dependent association of GRB2 with anti-Shc immunoprecipitates in LTR-EGFR/ErbB-3 cells (Fig. 7B, lane 6) and in LTR-EGFR cells (lane 4), significantly exceeding that of LTR-neo controls (lane 2). This finding was not due to variation of GRB2 expression levels as determined by direct immunoblot analysis of identical lysates (Fig. 7C) and thus indicated increased GRB2 association with Shc and possibly with the ErbB-3 cytoplasmic domain. Therefore, mitogenic signaling by the EGFR/ErbB-3 receptor involved coupling with both Shc and GRB2. Compelling evidence has recently

linked signaling by RTKs to the *ras* cytoplasmic pathway, implicating GRB2 as a bifunctional adapter molecule that can simultaneously bind tyrosine kinase substrates as well as the *ras* activator *sos*, which catalyzes guanine nucleotide exchange on the Ras protein (1, 10, 18, 32, 34, 51, 52, 57). Thus, our observations of EGF-dependent GRB2 and Shc recruitment by the chimeric receptor raise the possibility that the *ras* pathway participates in ErbB-3-mediated signal transduction.

DISCUSSION

The generation of chimeric receptors has facilitated elucidation of intracellular signaling pathways of RTKs for which ligands are not available. Such an approach has been applied in this study to the analysis of ErbB-3-mediated intracellular signal transduction. An EGFR/ErbB-3 chimera displayed two receptor classes with EGF binding affinities comparable to those of the wild-type EGFR. This provided the basis to quantitatively compare the biological and biochemical properties of these two kinases. Once cell lines expressing comparable receptor numbers were selected, the activities of the ErbB-3 and EGFR kinases could be compared with respect to the ability to couple with intracellular signaling pathways.

When expressed in NIH 3T3 cells, the EGFR/ErbB-3 chimera was able to transduce a mitogenic signal similar in magnitude to that elicited by the wild-type EGFR. These findings paralleled previous observations that EGF promoted anchorage-independent growth of LTR-EGFR/ErbB-3 transfectants but not of LTR-neo cells (27). Although NIH 3T3 cells are devoid of endogenous ErbB-3 protein (27), our results indicate that they must nonetheless possess essential components of its signaling pathway. The 50% effective dose for EGF-induced mitogenic response by EGFR/ErbB-3 was estimated from repeated experiments to range between 20 and 100 pM, a value equal to or lower than those reported for EGFR and the EGFR/ErbB-2 chimera (7). At such a ligand concentration, only several thousand EGFR/ErbB-3 molecules would be occupied at equilibrium, indicating efficient coupling of the chimeric receptor with intracellular signaling pathways.

Analysis of known components of transduction pathways indicated a strong association of the active ErbB-3 kinase with PtdIns 3-kinase. The efficiency of ErbB-3 in the recruitment of PtdIns 3-kinase activity was an order of magnitude greater than that of the EGFR and comparable to that of the PDGFR. Of note, ErbB-2 has been reported to form a complex with PtdIns 3-kinase at quantitatively similar levels as the EGFR (44). Thus, the potent coupling of ErbB-3 with this signaling pathway to date is unique among type I RTKs, functionally distinguishing ErbB-3 from related ErbB family members.

Recruitment of PtdIns 3-kinase activity has been linked to the ability of the p85 SH2 domains to bind specific phosphorylated tyrosine residues. On the basis of the affinity of specific phosphopeptides for a recombinant p85 SH2 domain, the consensus sequence for optimal p85 binding has been determined as P-Tyr–(Met/Val/Glu)–X–Met (14, 69). This peptide sequence occurs in six positions of the ErbB-3 cytoplasmic domain, including two regions that have been predicted as autophosphorylation sites (28, 69). Intriguingly, this consensus sequence (Tyr-Met-Ile-Met) is present in analogous positions of the EGFR and ErbB-2 tyrosine kinase domains. However, lack of autophosphorylation at this site in EGFR and ErbB-2 proteins (9, 19, 38) might explain the relatively lower coupling efficiency of PtdIns 3-kinase with these receptors. Indeed, a recombinant p85 SH2 domain has been shown to exhibit a 12-fold-lower binding affinity for the EGFR than for the PDGFR (22).

In spite of strong association of PtdIns 3-kinase with the phosphorylated EGFR/ErbB-3, we were unable to detect tyrosine phosphorylation of p85 upon EGF triggering. This finding is in accord with reports of PDGF activation of PtdIns 3-kinase activity in which p85 tyrosine phosphorylation was detected only when this protein was recombinantly overexpressed (22). These observations suggest that p85 tyrosine phosphorylation might occur at low stoichiometry, escaping detection by P-Tyr antibodies at physiological expression levels. Alternatively, its tyrosine phosphorylation may represent a transient phenomenon that is followed by rapid and specific dephosphorylation. Whereas the role of p85 tyrosine phosphorylation for enzymatic activity in vivo remains to be determined, the strong association of PtdIns 3-kinase activity with anti-P-Tyr immunoprecipitates appears to be due to coimmunoprecipitation with the phosphorylated receptor rather than p85 phosphorylation itself.

Two additional enzymes, PLCγ and GAP, have been implicated in the transduction of mitogenic signals (11, 23, 25, 36, 39, 41, 65). These enzymes do not appear to play a major role in ErbB-3 signaling. Such results further underscore the differences between ErbB-3 and other ErbB/EGFR family members which are quantitatively and qualitatively indistinguishable in the ability to couple with PLCγ or GAP (16). Moreover, differences in mitogenic potency of EGFR and ErbB-2 have implicated additional substrates in the signal transduction by these receptors (7, 15). These differences are apparently not dependent on their ability in coupling with the transforming protein Shc, since both EGFR and ErbB-2 have been shown to utilize Shc as a substrate (45, 56). In addition, we provide evidence that the ErbB-3 cytoplasmic domain recruits Shc and GRB2, suggesting that also the third member in this receptor subfamily may signal through the *ras* pathway as has been established for the EGFR, PDGFR, and insulin receptor (1, 10, 18, 32, 34, 51, 52, 57).

The biological response to a growth factor is determined by the ability of the corresponding receptor to activate specific signaling pathways. A recent study by Valius and Kazlauskas suggests that either PLCγ or PtdIns 3-kinase is necessary for PDGF-mediated DNA synthesis (62). Preferential ErbB-3 signaling through PtdIns 3-kinase is rather reminiscent of the signaling properties of colony-stimulating factor 1 and insulin receptors (3). For these receptors, no significant mitogenic signaling through PLCγ can be postulated. From evidence that insulin receptor represents an inefficient mitogenic signal transducer in fibroblasts (29, 50) and, like ErbB-3, recruits PtdIns 3-kinase, Shc, and GRB2 (1, 3, 57), it is probable that additional pathways have to participate in mitogenic signal transduction by RTKs such as ErbB-3 or colony-stimulating factor 1 receptor. In this context, it is of interest that we observed a doublet of tyrosine-phosphorylated proteins in the 95-kDa range in EGF-triggered LTR-EGFR/ErbB-3 transfectants. These proteins did not appear phosphorylated in response to EGFR activation. Molecular characterization of such putative substrate proteins will be required to determine their role in ErbB-3-mediated mitogenesis.

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