Mammalian phospholipase D (PLD) activity becomes up-regulated when cells are stimulated by a variety of hormones, growth factors, and other extracellular signals. Two distinct PLDs, PLD1 and PLD2, have been identified. The mechanism through which each PLD is activated, however, is poorly understood. Using transiently transfected human embryonic kidney fibroblasts (HEK293), we demonstrate here that PLD1 activity, and to a lesser extent PLD2 activity, is stimulated in response to epidermal growth factor (EGF). PLD2, but not PLD1, associates with the EGF receptor in a ligand-independent manner and becomes tyrosine-phosphorylated upon EGF receptor activation. Tyrosine 11 (Tyr-11) of PLD2 was identified as the specific phosphorylation site. Mutation of this residue to phenylalanine enhanced basal activity almost 2-fold, but did not alter the magnitude of the EGF-mediated increase in PLD2 activity. In conclusion, we show here for the first time agonist-stimulated activation of both PLD1 and PLD2 in vivo and provide evidence of a distinct type of interaction for each isoform with the EGF receptor. Moreover, our results suggest that agonist-induced tyrosine phosphorylation plays a role in PLD2 regulation.

Phospholipase D (PLD) activity is stimulated by a great variety of extracellular agonists and has been suggested to regulate signal transduction as well as cellular functions such as membrane trafficking and cytoskeletal reorganization (reviewed in Refs. 1–3). PLD hydrolyses phosphatidylcholine (PC), thereby generating choline and the second messenger phosphatidic acid (PA). PA in turn can be further metabolized by phospholipase A₂ to lysophosphatidic acid (LPA) and by phosphatidic acid phosphohydrolase (PAP) to diacylglycerol (DAG) (reviewed in Refs. 2 and 4). Cell physiological roles for PA have been investigated extensively and specific targets include protein kinases (5–8), protein tyrosine phosphatase (9), phosphatidylinositol-4,5-bisphosphate (PIP₂), and oleate (reviewed in Refs. 1–3, and 16). Furthermore, tyrosine kinase-mediated PLD activation has been observed in several cell systems (reviewed in Ref. 17). The activation of PLD by EGF in pancreatic acinar cells can be inhibited by the tyrosine kinase inhibitor genistein, indicating that tyrosine phosphorylation of unknown proteins is an essential feature for the stimulation of at least some PLD activities (18). Similarly, 4-hydroxynonenal-induced PLD activation in vascular endothelial cells is inhibited by tyrosine kinase inhibitors and enhanced by protein tyrosine phosphatase inhibitors (19).

Two PLDs have been cloned and characterized: PLD1 (20) and PLD2 (21). PLD1 has low basal activity, requires PIP₂ for activity, and is activated in vitro by PKCα, ARF, and Rho (20, 24). PLD1 interacts with RalA (22) and can be inhibited by oleate (20). PLD1 is located primarily in perinuclear regions in rat embryo fibroblasts (21) and can promote nascent secretory vesicle release (23). Two splice variants of PLD1 have been identified, though no differences either in regulation or activity have been observed thus far (24). Tyrosine phosphorylated PLD1 has been observed in HL-60 granulocytes (25), however, whether this is of importance for regulation of PLD1 activity or subcellular localization remains to be determined.

The PLD1 and PLD2 amino acid sequences share ~52% identity. PLD2 is associated with the plasma membrane and has, in contrast to PLD1, high basal activity which is not further activated by PKCα, ARF, or Rho in vitro (21). Its mode of regulation in vitro at normal levels of expression is not known, although it is probably held quiescent unless stimulated. Upon serum stimulation, PLD2 undergoes redistribution and a role in cytoskeletal reorganization has been suggested (21).

Ligand-induced activation of the EGF receptor has been shown to stimulate PC hydrolysis leading to increasing PA concentration in a variety of cell systems (26–31). Whether this is mediated by PLD1 or/and PLD2, or yet unidentified PLD...
isoforms has not been determined. In this paper, we provide evidence that EGF treatment stimulates PLD1 and PLD2 activity in human embryonic kidney 293 (HEK293) cells. PLD2, but not PLD1, forms a physiological complex with the EGF receptor and is tyrosine-phosphorylated upon receptor activation. Whereas EGF receptor kinase activity is essential for the stimulation of PLD2 activity, tyrosine phosphorylation of PLD2 is not.

**EXPERIMENTAL PROCEDURES**

cDNAs—pCGN expression vectors containing the cDNAs of human PLD1 (hPLD1) and mouse PLD2 (mPLD2) have been described previously (20, 21, 32). pCGN additionally encodes an influenza-epitope (HA)-tag which becomes expressed in-frame at the N terminus of the PLDs (the resulting proteins are denoted as HA-PLD1 and HA-PLD2). The cDNAs for the EGF receptor and mPLD2 (without an HA-tag) were also cloned into a cytomegalovirus promoter-enhancer-driven expression vector. Deletion of the cytosolic domain of EGFR amino acids 655–721 was generated using the Muta-Gene M13 mutagenesis kit (Bio-Rad) and the following mutagenesis primers: PLD2 Y11F, 5'-GGTAATT-CAGTCAGTCG-3'; PLD2 Y11F reverse, 5'-GGGTTCAAGAATGGCCATAGG-3'; PLD2 Y11F, 5'-GGGTTCAAGAATGGCCATAGG-3'; PLD2 Y11F reverse, 5'-GGGTTCAAGAATGGCCATAGG-3'. The PLD2 Y11F mutant was then subcloned back into the HA-tag expression vector. Deletion of the cytosolic domain of EGFR amino acids 357–365 of the EGFR was used. Anti-influenza (HA)-tag antiserum produced by immunization with a synthetic peptide encoding the 15 C-terminal amino acids 655 of the EGFR was used. Anti-PLD1 and anti-PLD2 antibodies were generated through enzymelinked full-length EGFR cDNA and subsequent introduction of a stop codon at amino acid 655.

Cell Culture—HEK293 cells, which express undetectable endogenous EGFR levels, were obtained from A. Ullrich, Martinsried. The cells were cultured in Dulbecco’s modified Eagle’s medium containing high glucose, 0.5% fetal calf serum and 10 mM l-glutamine, 100 units/ml penicillin (Life Technologies, Inc.) at 37 °C in a 5% CO2 enriched, humidified atmosphere. The cells were starved in Dulbecco’s modified Eagle’s medium containing high glucose, 0.5% fetal calf serum, and 2 mM l-glutamine, 10 mg/ml streptomyacin, and 100 units/ml penicillin (Life Technologies, Inc.) overnight prior to initiating the 30-min EGF (50 nM) stimulation.

**Transfection and Transient Expression**—Transient transfection of HEK293 cells was performed essentially as described by Chen and Okayama (33) and Gorman et al. (34).

**Cell Lysis, Immunoprecipitations, and Gel Electrophoresis**—Prior to lysis, the cells were treated with EGF (50 ng/ml) for 30 min and washed once with phosphate-buffered saline. Cells were lysed in 0.4 ml per 3-cm dish with ice-cold lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 20 mM NaF, 200 μM Na3VO4, 1 mM AEBSF (Calbiochem), 1 mg/ml p-nitrophenyl phosphate (Sigma)) and pre cleared by centrifugation at 15,000 × g for 10 min at 4 °C. Immunoprecipitations were performed by addition of 30 μl of Protein G-Agarose (Boehringer-Mannheim) and the appropriate antibody for 3 h, followed by brief centrifugation. The precipitates were washed three times in washing-buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 20 mM NaF, 200 μM Na3VO4, 1 mM AEBSF, 1 mg/ml p-nitrophenyl phosphate). For gel electrophoresis, the immunoprecipitates were dissolved in 2X Laemmli buffer (35), and the proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell, BA85). The immunoreactive proteins were visualized using horseradish-peroxidase-coupled secondary antibodies and enhanced chemiluminescence reagents according to the manufacturer instructions (Amersham Pharmacia Biotech).

**Antibodies**—Polyclonal rabbit antiserum against PLD2 was produced by immunization with a synthetic peptide encoding the 15 C-terminal amino acids of mPLD2 (CPLDEKQTTMFVIR). The antiserum recognizes both hPLD1 and mPLD2. Anti-phosphotyrosine (PY20) antibody was obtained from Transduction Laboratories, and anti-EGFR antibody for immunoprecipitation was from Merck, Darmstadt. For detection of the EGFR by Western blot analysis, polyclonal rabbit antiserum produced by immunization with a synthetic peptide encoding amino acids 357–365 of the EGFR was used. Anti-influenza (HA)-tag monoclonal antibody was obtained from Boehringer Mannheim.

**PLD Assays**—In vivo measurements of PLD activity were performed according to Wakelam, M. J. O. et al. (36), with the following modifications. PLD activity was assayed by measuring the accumulation of 14C-labeled phosphatidylbutanol (PtdBut). The cells were labeled with 4 μCi 14C-palmitic acid per 2-cm dish added to the starvation medium and incubated overnight at 37 °C in a 5% CO2 enriched, humidified atmosphere. The cells were washed twice in HHBB buffer (1.26 mM CaCl2, 0.5 mM MgCl2, 0.4 mM MgSO4, 5.37 mM KCl, 137 mM NaCl, 4.2 mM NaH2PO4, 10 mM Hepes, pH 7.4, 1% (w/v) bovine serum albumin, 10 mM glucose) and incubated in 0.3% butanol-1-ol in HHBB buffer for 20 min at 37 °C. Subsequently, the cells were treated with or without 50 ng/ml EGF in HHBB containing 0.3% butanol-1-ol for an additional 30 min. After the incubation, the buffer was aspirated and 0.5 ml of ice-cold methanol was added to each well. The cell debris was scraped into a glass vial and kept on ice. Chloroform and H2O were added to give a final CHCl3:CH3OH:H2O ratio of 1:1:0.8 (v/v). After vortexing, the vials were centrifuged at 1200 × g for 5 min, and the organic phase was spotted on Whatman LK5DF thin-layer chromatography plates. The labeled products were separated by thin-layer chromatography using...
PLD2 forms a physical complex with EGFR. HEK293 cells were transiently transfected with EGFR (lanes 1 and 2), HA-PLD2 (lanes 3 and 4), EGFR and HA-PLD1 (lanes 5 and 6), EGFR and HA-PLD2 (lanes 7 and 8), EGFR K721A and HA-PLD2 (lanes 9 and 10), or with ΔEGFR (which encodes the N-terminal 655 amino acids) and HA-PLD2 (lanes 11 and 12) expression plasmids. The cells were either left untreated or were stimulated with EGF for 30 min and subsequently lysed. The EGFR was immunoprecipitated from all samples (using anti-EGFR antibodies that recognize the extracellular domain of the EGFR), and the proteins were separated by SDS-PAGE. Immunoprecipitation of the EGFR and co-immunoprecipitation of PLD2 was determined by Western blot analysis using aHA-tag-specific (PLD2, upper panel) and EGFR-specific antibodies (upper middle panel), respectively. The expression of PLD2 and tyrosine phosphorylation of proteins was determined by Western blot analysis of total cell lysates using aHA-tag-specific antibodies (aHA, lower middle panel) to detect HA-PLD1 and HA-PLD2 and phosphotyrosine-specific antibody PY20 (aPtyr, lower panel). Proteins were visualized using horseradish peroxidase-coupled secondary antibodies and the ECL™ (Amersham Pharmacia Biotech) detection method.

RESULTS

EGF-induced Activation of PLD1 and PLD2 in Intact Cells—EGF treatment has been previously shown to activate PLD activity in various cell systems (26–31). To investigate whether the recently cloned isoforms PLD1 and PLD2 are targeted by the EGFR, we transiently overexpressed either Flu epitope-tagged PLD1 (HA-PLD1) or Flu epitope-tagged PLD2 (HA-PLD2) in combination with the EGFR into HEK293 cells. Activation of PLD was determined by the unique ability of PLD to produce, in the presence of butanol, phosphatidylbutanol (PtdBut) and total lipids. In vitro PLD activity was determined by measuring the release of 3H-choline from didecanoyl phosphatidyl[3H]choline (Amersham Pharmacia Biotech) according to Vinggaard et al. (37).

EGF-induced Tyrosine Phosphorylation and Activation of PLD2

PLD2 becomes tyrosine phosphorylated in response to EGF. The experiment in Fig. 1 showed that both PLD1 and PLD2 become activated upon EGF-induced activation of the intrinsic tyrosine kinase activity of the EGFR. To investigate whether PLD1 or/and PLD2 served as a substrate for the EGFR, HA-PLD1 and HA-PLD2 were co-expressed with the EGFR, following which the cells were either left untreated or were treated with EGF for 30 min. Immunoprecipitation of the upper phase of a mixture of 2,2,4-trimethylpentane:ethyl acetate:acetic acid:water ratio of 50:110:20:100 (v/v). PhosphorImager technology (Molecular Dynamics) was used to determine the amount of labeled PtdBut and total lipids. In vitro PLD activity was determined by measuring the amount of labeled PtdBut and total lipids. The stimulation of cells with EGF was carried out for 30 min because time course experiments in HER293 cells transiently transfected with HA-PLD2, and the EGFR revealed maximal PLD2 activation after 30 min of EGF stimulation (data not shown).

Similar to native HEK293 cells, overexpression of the EGFR did not result in elevated PLD activity in cells that were treated with EGF for 30 min (Fig. 1), demonstrating that endogenous levels of PLD are either relatively low or unresponsive to EGFR treatment. Overexpression of PLD2 resulted in very high basal activity (up to 10-fold higher than in untransfected HEK293 cells), which was further stimulated (1.3-fold) by EGF treatment. Overexpression of PLD2 and the EGFR led to a further increase in EGF-induced PLD2 activity (1.7–2.1-fold in individual experiments), whereas the high basal activity was unaffected. The increase in PLD2 activity did not result from the presence of the HA-tag since a similar increase was observed for PLD2 lacking the HA-tag (see Fig. 6).

Tyrosine kinase activity has been shown to be essential for EGF-induced PLD activation in pancreatic acinar cells (18). To investigate the importance of the EGFR tyrosine kinase activity for PLD2 activation, we overexpressed HA-PLD2 together with a kinase-inactive EGFR mutant (EGFR K721A). EGF treatment of cells expressing EGFR K721A and PLD2 did not result in activation of PLD2, clearly showing that tyrosine kinase activity is an essential feature for the activation of PLD2 (Fig. 1).

Overexpression of HA-PLD1 and the EGFR revealed that PLD1, in contrast to PLD2, has low basal activity, although the expression levels of PLD1 and PLD2 are, upon transient transfection into HEK293 cells, comparable (see Fig. 2). Similar to PLD2, EGF treatment enhanced PLD1 activity significantly (2.8-fold), demonstrating that both PLD1 and PLD2 are activated by the EGFR (Fig. 1).
PLD1 and PLD2 with HA-tag-specific antibodies and subsequent Western blot analysis with phosphotyrosine-specific antibodies revealed EGF-induced tyrosine phosphorylation of PLD2, but no tyrosine phosphorylation of PLD1 (Fig. 2). These data show that PLD2 is a substrate for a tyrosine kinase activated by EGF stimulation, which could be the EGFR itself or a different kinase. Confirmation that the PLD2 tyrosine phosphorylation requires activation of the EGFR pathway was demonstrated using the kinase-inactive EGFR (EGFR K721A) under the same conditions, which did not lead to detectable PLD2 phosphorylation (Fig. 3, lanes 9 and 10).

PLD2 Forms a Physical Complex with the EGFR—Because PLD2 is tyrosine phosphorylated in an EGFR-dependent manner and has been shown to be localized to the plasma membrane, we investigated whether PLD2 and the EGFR interact. Complex formation was analyzed by Western blot analysis after immunoprecipitation of the EGFR (using a monoclonal anti-EGFR antibody which recognizes the extracellular domain of the receptor) from HA-PLD2 and EGFR co-expressing HEK293 cells. As shown in Fig. 3, immunoprecipitation of the wild-type EGFR resulted in co-immunoprecipitation of PLD2 (Fig. 3, lanes 7 and 8, upper panel). The amount of PLD2 that was co-immunoprecipitated with the receptor was similar in untreated and EGF-treated cells, indicating that the interaction does not require receptor activation. This is supported by the finding that PLD2 also interacts with the kinase-inactive EGFR (EGFR K721A) (Fig. 3, lanes 9 and 10). The complex formation between PLD2 and EGFR was eliminated when the cytosolic part of the receptor was almost completely deleted (ΔEGFR, which encodes amino acids 1–655) (Fig. 3, lanes 11 and 12), suggesting not unexpectedly that the interaction is mediated by the cytosolic part of the EGFR. In contrast to PLD2, PLD1 did not co-immunoprecipitate with the EGFR either in EGF-treated or in unstimulated cells (Fig. 3, lanes 5 and 6). EGF-induced activation of the EGFR as well as EGFR-specific tyrosine phosphorylation was confirmed by Western blot analysis using the phosphotyrosine-specific antibody PY20 (Fig. 3, lower panel). We conclude from these data that PLD2, but not PLD1, forms a physical complex with the EGFR that is independent of receptor activation.

The interaction of PLD2 with the EGFR was confirmed through determination of EGFR-bound PLD activity in vitro. As shown in Fig. 4, PLD activity co-immunoprecipitated with the EGFR from cells that were co-transfected with HA-PLD2 and EGFR expression plasmids, whereas immunoprecipitation of the EGFR from cells transfected only with HA-PLD2 resulted in recovery of very little PLD activity (Fig. 4, left panel). The PLD2 activity in the EGFR complex did not increase upon EGF stimulation, whereas total PLD activity immunoprecipitated with HA-tag antibodies increased about 1.7-fold upon EGF stimulation (Fig. 4, right panel). Whether this is because of ligand-induced receptor internalization and degradation, partial release of PLD2 from the complex upon EGF stimulation, or changes in PLD2 activity is currently not known, although the first possibility appears most likely because total PLD2 activity increases and similar results are seen in Fig. 1. Even though we do not know the molar ratios of the EGFR and PLD2 upon overexpression in HEK293 cells, the activity data suggest that only a minor fraction of PLD2 (about 10%) is complexed with the EGFR in this setting.

Because co-factors which activate PLD2 under in vitro conditions have not been identified yet, tyrosine phosphorylation of PLD2 may have an important regulatory function. To investigate whether tyrosine phosphorylation is required for activation of PLD2, we attempted to identify the specific tyrosine residue(s) in PLD2 which is(are) phosphorylated through the action of the activated EGFR. Comparison of the amino acid sequences of human PLD1 and mouse PLD2 showed that four tyrosine residues in PLD2 (Tyr-11, Tyr-14, Tyr-165, and Tyr-470) are not conserved in PLD1. These tyrosine residues were mutated to phenylalanine, and the ability of the EGFR to phosphorylate these mutants on tyrosine was investigated upon co-overexpression in HEK293 cells. As shown in Fig. 5A, mutation of tyrosine 11 to phenylalanine (Y11F) completely abolished EGF-induced tyrosine phosphorylation of PLD2. None of the other mutations impaired EGF-induced tyrosine phosphorylation of PLD2, showing that tyrosine 11 is the only tyrosine phosphorylation site. It should be noted that none of the mutations had any effect on the protein stability as shown in Fig. 5A, lower panel. We then sought to determine whether the Y11F mutation interferes with the ability of PLD2 to associate with the EGFR, which might be essential for EGF-induced tyrosine phosphorylation of PLD2. HA-PLD2 and PLD2 Y11F were co-expressed with the EGFR in HEK293 cells, and the amount of PLD2 and PLD2 Y11F that co-immunoprecipitated with the EGFR was determined by Western blot analysis using antiserum directed against the C-terminal 15 amino acids of PLD2. As shown in Fig. 5B, similar amounts of both wild-type PLD2 and PLD2 Y11F co-immunoprecipitated with the EGFR, revealing that differences in the affinity for the EGFR do not account for the inability of the EGFR to phosphorylate PLD2 Y11F.

The effect of the Y11F mutation on EGF-induced PLD2 activation was subsequently studied in HEK293 cells after overexpression of wild-type and PLD2 Y11F. As shown in Fig. 6, both wild-type and PLD2 Y11F activity were stimulated upon EGF treatment to the same extent (1.7- and 1.8-fold, respectively), although the basal and stimulated activities of PLD2 were both elevated as a consequence of the Y11F mutation. These data indicate that the EGF-induced tyrosine phosphorylation of PLD2 is neither essential for activation of the enzyme nor for the association with the EGFR and suggests that it may have an important role in yet unidentified functions.
result of receptor stimulation. Because overexpressed PLD2 Y/F mutants was determined by incubation with anti-phosphotyrosine-nitrocellulose filters. Tyrosine phosphorylation of PLD2 and the PLD2 min. Total cell lysates were separated by SDS-PAGE and transferred to which were left either untreated or were stimulated with EGF for 30 min. Transiently transfected together with the EGFR into HEK293 cells blot analysis using anti-PLD2-specific antiserum (21). The fold increase of PtdBut formation in response to EGF over basal is given for each set of the transfections. Data are means ± S.E. of one experiment performed in triplicate. The result shown is representative of three independent experiments.

Fig. 5. The EGFR phosphorylates PLD2 at tyrosine 11. A, wild-type PLD2 (lacking the HA-tag) as well as PLD2 Y/F mutants were transiently transfected together with the EGFR into HEK293 cells which were left either untreated or were stimulated with EGF for 30 min. Total cell lysates were separated by SDS-PAGE and transferred to nitrocellulose filters. Tyrosine phosphorylation of PLD2 and the PLD2 Y/F mutants was determined by incubation with anti-phosphotyrosinespecific antibody PY20 (αPTyr, upper panel), and their expression was confirmed by incubation with PLD2-specific antisera (αPLD, lower panel). B, HEK293 cells expressing either the EGFR and HA-PLD2 or the EGFR and PLD2 Y11F were left untreated or were treated with EGF for 30 min and lysed. The EGFR was immunoprecipitated (using anti-EGFR antibodies that recognize the extracellular domain of the EGFR), and the proteins were separated by SDS-PAGE. Co-immunoprecipitation of HA-PLD2 and PLD2 Y11F was assessed by Western blot analysis using anti-PLD2-specific antisera (αPLD2). Proteins were visualized using horseradish peroxidase-coupled secondary antibodies and the ECL™ (Amersham Pharmacia Biotech) detection method.

DISCUSSION

The stimulation of PLD activity has been observed in various cell types in response to a large number of agonists including growth factors. EGF treatment stimulates PLD activity in Swiss 3T3 (26, 29, 31), A431 (28, 30), and rat fibroblasts (27); however, neither the mechanism of activation nor which of the PLD isoforms are activated has been determined. Our investigations show that both human PLD1 and mouse PLD2 are activated upon EGFR stimulation in HEK293 fibroblasts. The fold induction of PLD1 and PLD2 activity corresponds well to the increase of endogenous PLD activity found in response to EGF in other cell systems (26, 28, 30). Similar to the in vitro activity in COS-7 cells (21), PLD1 has low and PLD2 high basal activity in HEK293 cells. Because PLD2 has high basal activity in vitro, it has been suggested that in vivo it is normally inhibited through one or more mechanisms (38) and that it is de-repressed when receptors undergo stimulation (21). Although our data do not discriminate between this hypothesis and simple activation, the 2-fold induction of PLD2 activity that we observed upon EGFR treatment does demonstrate that PLD2 activity is regulated in vivo and that it increases as a result of receptor stimulation. Because overexpressed PLD2 may overwhelm normal regulatory mechanisms (21), the degree of EGF-mediated activation of PLD2 may be much more dramatic for endogenous PLD2. The activation of PLD by EGF in pancreatic acinar cells has been shown to be sensitive to the tyrosine kinase inhibitor genistein, indicating that the intrinsic tyrosine kinase activity of the receptor is an essential feature for activation of at least some PLD activities (18). We show here, that indeed the activation of PLD2 requires EGFR tyrosine kinase activity (Fig. 1). Whether this also applies to PLD1 remains to be investigated. PLD2, but not PLD1, forms a physical complex with the EGFR and is tyrosine-phosphorylated in response to receptor activation. The interaction between the EGFR and PLD2 is mediated by the intracellular part of the receptor (Fig. 3). Further mapping of the interaction site using various EGFR deletion mutants suggests that several subdomains of the cytosolic part mediate the interaction with PLD2.2 Taken together, the findings that PLD2 complexes with the EGFR and that it becomes tyrosine-phosphorylated in an EGF and EGFR-dependent manner suggests as the most likely possibility that PLD2 is a direct substrate for the EGFR. Nonetheless, attempts to show interaction between the EGFR and PLD2 under in vitro conditions failed.2 Whether this is because of a certain receptor confirmation which is stabilized by the plasma membrane in vivo, or to formation of a multi-protein complex which is not properly formed under in vitro conditions, is not clear at present. It is, however, interesting to note that the interaction between the EGFR and PKCα (39) has very similar properties.2 The identification of tyrosine 11 as the specific phosphorylation site in PLD2 and its subsequent mutation to phenylalanine clearly showed that tyrosine phosphorylation of PLD2 is not required for its basal activity or for agonist-dependent stimulation. Tyrosine phosphorylation of PLD1 has been shown recently in human granulocytes in response to the protein tyrosine phosphatase inhibitor pervanadate (25). Whether this affects PLD1 activity has not been determined. We have not been able to detect any tyrosine phosphorylation of PLD1 in response to EGF treatment suggesting the involvement of other tyrosine kinases in PLD1 phosphorylation in the presence of protein tyrosine phospho-

2 R. Slaaby and K. Seedorf, unpublished observation.
tase inhibitors. Furthermore, because tyrosine 11 is not conserved in the amino acid sequence of PLD1, it must be phosphorylated at a distinct site.

The total PLD2 activity increased by approximately 2-fold in response to EGF, whereas the receptor-associated activity did not increase. These data may indicate that PLD2 is released from the receptor upon stimulation and translocates to another location. Several different mechanisms might underlie the PLD2 stimulation, such as increase in PIP₂, either locally or as a result of translocation. In this context, the increased basal and stimulated activity of PLD2 Y11F suggests that the mutant enzyme is in a more favorable local environment and/or is more active than the majority of the wild-type PLD2, which presumably is not phosphorylated at Tyr-11. In this context it is interesting to note that PA phosphohydrolase (PAP), which is presumably not phosphorylated at Tyr-11. In this context it is interesting to note that PA phosphohydrolase (PAP), which converts PLD-generated PA to DAG, also has been shown to co-immunoprecipitate with the EGFR. Following EGF activation, PAP was released from the receptor complex and associated with PKCε (40). Whether PLD2 translocates in a similar way remains to be investigated.

The in vitro characterization of PLD1 and PLD2 has revealed that PLD1 is activated by ARF, PKCα, and Rho in a synergistic manner, whereas PLD2 activity remains unaffected by these co-factors (21). How the EGFR mediates the activation of either PLD1 or PLD2 in vivo has not been determined yet. The specific interaction with, and tyrosine phosphorylation of PLD2 by the EGFR, as well as the specific subcellular localization of PLD1 and PLD2 (21) suggests distinct mechanisms of activation.

The EGFR receptor is just one of many tyrosine kinase receptors found in cells. Our findings raise the possibility that PLD2 expressed at endogenous levels may largely or entirely be complexed with the plasma membrane localized receptors. In the unstimulated levels, such receptors tend to be sequestered into relatively quiescent regions of the membrane that are deficient in PIP₂. Because PIP₂ is a required co-factor for PLD2, this might provide a potential mechanism through which cells could keep PLD2 inactive in the absence of agonist stimulation. Alternatively, other components of the complex might contribute to the PLD2 inhibition. Subsequent receptor stimulation would lead to new generation of PIP₂ through PLC activation, and potentially to PLD2 translocation to more active membrane sites (either plasma membrane or vesicles).

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