The epidermal growth factor receptor (EGFR) plays a critical role in the development, proliferation, and differentiation of cells of epithelial and mesenchymal origin. These EGFR-dependent cellular processes are mediated by a repertoire of intracellular signaling pathways triggered by the activation of the EGFR cytoplasmic domain, which originates from ligand binding of its extracellular domain. To understand the molecular mechanisms by which the intracellular domain of EGFR transmits mitogenic messages to the downstream signaling pathways, we used the cytoplasmic region of EGFR as bait in yeast two-hybrid screening. We found that ADP-ribosylation factor 4 (ARF4) interacts with the intracellular part of EGFR and mediates the EGF-dependent cellular activation of phospholipase D2 (PLD2) but does not mediate the activation of PLD1. In addition, ARF4-mediated PLD2 activation leads to dramatic activation of the transcription factor activator protein 1 (AP-1), and, importantly, ARF4 activity is required for EGF-induced activation of cellular AP-1. Our findings indicate that ARF4 is a critical molecule that directly regulates cellular PLD2 activity and that this ARF4-mediated PLD2 activation stimulates AP-1-dependent transcription in the EGF-induced cellular response.

Overexpression of EGFR and EGF, as well as constitutive activation of mutant EGFR, give rise to the deregulation of this EGFR-dependent signaling network, which is involved in the development and malignancy of numerous types of human cancers, including cancer of the head and neck, lung, bladder, breast, colon, prostate, kidney, ovary, brain, and pancreas (1). The EGFR receptor is the prototypical receptor with intrinsic protein tyrosine kinase activity (2–4). Upon ligand binding by its extracellular domain, EGFR forms homodimers or heterodimers with the closely related ErbB receptors (such as ErbB2/Her2) and begins tyrosine autophosphorylation of its cytoplasmic domain (2–4). This autophosphorylation of the intracellular domain of EGFR is critical for signal transduction, since it creates docking sites for various signaling molecules, such as molecules containing Src homology 2 or phosphotyrosine binding domains. These molecules create signaling complexes that lead to oncogenic responses by linking EGFR activation to numerous cytoplasmic signaling pathways (2–4). In cancer cells, the signaling domain of EGFR mediates and regulates the proliferative signals generated from this receptor. Therefore, identifying molecules that interact with the signaling domain of EGFR will contribute not only to the elucidation of the regulatory mechanism of EGFR in cancer progression but also to the development of new treatments for uncontrolled growth of human cancers.

To further understand the molecular mechanisms by which the intracellular region of EGFR transmits signals to various intracellular signaling pathways, we used the cytoplasmic domain of EGFR as bait in yeast two-hybrid screening of a human carcinoma cDNA library. Here we identify an intracellular protein ARF4 that interacts with the cytoplasmic domain of EGFR. ARFs, members of the Ras superfamily, are small 20-kDa guanine nucleotide-binding proteins. The inactive GDP-bound form of ARF is soluble, although it can weakly associate with the cell membrane. On the other hand, the active GTP-bound form binds tightly to the membrane (5). On the basis of deduced amino acid sequences and phylogenetic analysis, ARFs have been divided into three classes: class I (ARF1, -2, and -3), class II (ARF4 and -5), and class III (ARF6) (6). Although all three classes of ARF proteins are capable of activating cellular phospholipase D, the specific roles of each ARF seem to be different. Class I is involved in the trafficking pathway that links the endoplasmic reticulum, Golgi, and endosomal systems, and class III, instead, takes part in the trafficking pathway that links the endosome and plasma membrane system (7). Many researchers believe that class II ARFs are only supplementary to class I ARFs and, as such, have focused their attention on class I ARFs while nearly ignoring the cellular functions of the class II ARF4 and ARF5 proteins. Here, we show that EGFR does not activate PLD1 but does activate cellular PLD2 through the activation of ARF4. Moreover, this ARF4-dependent activation of PLD2 is required for inducing the EGFR-induced transcriptional activity of activator protein 1 (AP-1).

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Screening—The EcoRI fragment encoding the cytoplasmic domain of EGFR was fused in frame with the GAL4 DNA binding domain (BD) of the pGBT7 fusion vector (Clontech) to create the BD/EGFR construct. A human epithelial carcinoma cDNA library was separately fused with the GAL4 activation domain (AD) of the pGAD-GH vector (Clontech). The yeast strain pY69–2A was co-transformed, by a lithium acetate-based method, with this library and the plasmid.
BD/EGFR construct. The transformed yeast cells were screened, according to the manufacturer’s protocols, for their ability to grow on plates lacking histidine and adenine.

**DNA Constructs—PCR was used to obtain full-length cDNAs encoding ARF4, PLD1, PLD2, and EGFR by use of the Rapid-Screen™ arrayed human placenta cDNA library, which is constructed in the pCMV6-XLA (ORIGENE, Rockville, MD) vector. The ARF4 wild-type and mutant cDNA fragments were cloned either into the mammalian expression vector pEBG, which also encodes the glutathione S-transferase tag (8), or into the multiple cloning site of the vector pRK5, which encodes three copies of the hemagglutinin (HA) epitope at its carboxyl terminus (9). All mutations in the ARF4 gene were generated by PCR-based mutagenesis as previously described (9).**

**Cell Lines Culture Conditions—293T cells overexpressing EGFR (293T/EGFR) were obtained by transfecting, via the calcium phosphate method, the hygromycin selection vector pCMV6-XLA into 293T cells, which express undetectable levels of endogenous EGFR. Transfected cultures were selected with hygromycin (300 μg/ml) for 10–14 days at 37°C. All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone) as described previously (10). Cells were grown to subconfluence and then starved overnight by replacing medium with serum-free Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine and nonessential amino acids before initiating the 30-min stimulation with EGF (50 ng/ml).**

**Immuno precipitation—Cells were solubilized in lysis buffer containing 2 mM HEPES (pH 7.6), 1% Triton X-100, 137 mM NaCl, 0.1 mM Na3VO4, 25 mM β-glycero phosphate, 3 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride as described (10, 11). Cell lysates were then incubated with protein G-coupled Sepharose beads (Amersham Biosciences) for 1 h, after which the beads were discarded, and the supernatants were incubated with specific antibodies (2 μg/ml anti-EGF antibody or IgG from preimmune serum) for 1 h followed by overnight incubation with protein G-agarose at 4°C. Samples were collected and washed four times with lysis buffer. The precipitated samples were analyzed by Amersham Bioscience’s ECL blotting system with specific antibodies (200 ng/ml unless mentioned). The R891 anti-ARF4 antisera, which recognizes the C-terminal repeat of interferon γ-activated sequence (GAS), nuclear factor of activated T cells (NFAT), or serum response element (SRE). Cells were grown on 35-mm multwell plates (Nunc, Naperville, IL) and transiently transfected with 5 μg of total plasmid DNA as described previously (10, 11). Briefly, in each transfection, 0.05 μg of the β-galactosidase expression plasmid (pCMV-βGal, Clontech, Palo Alto, CA) was included to determine the transfection efficiency. At 24 h posttransfection, cells were starved overnight and treated with or without stimuli as described in the figure legends. Cells extracts were prepared, and the activities of β-galactosidase and luciferase were measured as described previously (10, 11). The luciferase activity in each experiment was divided by the activity of β-galactosidase to correct for transfection efficiency.**

**Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared as described (14). The AP-1 oligonucleotide was labeled with [γ-32P]ATP and incubated with nuclear extracts for 30 min by using the gel shift assay system kit (Promega, Madison, WI). The specificity of binding was determined by adding homologous or mutated unlabeled synthetic oligonucleotides (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For gel supershift assays, rabbit affinity-purified antibodies (2 μg) to Jun and Fos families of nuclear factors (Santa Cruz Biotechnology) were incubated for 30 min with nuclear extracts and with synthetic oligonucleotides at room temperature. DNA-protein complexes were resolved on nondenaturing, nonreducing 4% acrylamide gels, and the complexes were visualized by PhosphorImager technology.**

**RESULTS**

Identification of ARF4 as an EGFR-interacting Protein by Yeast Two-hybrid Screening—To identify molecules that act as mediators of EGFR-dependent signaling, we used the cytoplasmic domain of EGFR as bait in a yeast two-hybrid screen. From a human carcinoma cDNA library, 2 × 10⁷ transformants were screened, and 102 positive clones were selected for sequence analysis based on their potential interaction with EGFR. In this GAL4 system, growth of transformed yeast in medium lacking histidine and adenine is completely dependent on the presence of sequences from both ARF4 and EGFR (Fig. 1A). Using the BLAST algorithm and the nucleotide data base at the National Library of Medicine, we found one clone that encoded the small GTPase ARF4.

To study the interaction between EGFR and ARF4 in mammalian cells, we transfected expression vectors encoding either wild-type or mutant ARF4 with three C-terminal HA epitope tags (ARF4-HA3) into 293T/EGFR cells (Fig. 1B). These transfected cells were treated with or without EGF and later lysed to prepare total cell lysates. From these cell lysates, anti-HA antibody was used to immunoprecipitate HA-tagged ARF4s and other cellular molecules complexed with these ARF4s. The ARF4-HA3 complex was recovered by centrifugation, and the pellets were separated by SDS-PAGE and analyzed by Western blot with an anti-EGFR antibody. As shown in Fig. 1B, EGFR co-purified with wild-type ARF4 only in lysates from EGF-treated cells but not from cells without EGF stimulation. In contrast, EGFR treatment was not required for the co-purification of EGFR with the active form of the ARF4 mutant, ARF4(Q71L), which theoretically constitutively binds to GTP (Fig. 1B). The inactive form of ARF4, ARF4(T31N), which constitutively binds to GDP, did not co-immunoprecipitate with EGFR.
EGFR regardless of EGF stimulation of the cells (Fig. 1B). In order to test whether EGFR interacts with endogenous ARF4 or other members of the ARF family, extracts from cells stimulated with or without EGF were immunoprecipitated with anti-EGFR or control antibodies. The resulting immunocomplexes were analyzed by immunoblotting with R891 anti-ARF4.
Molecular Dynamics. The density of the ARF4 band in digitonin-treated cell pellets was set arbitrarily as 1. Without digitonin. On Western blots, the relative densities of each ARF4 band in SDS-PAGE and analyzed by immunoblot with the anti-HA antibody. The control sample shows results from cells treated with culture medium only, whereas, after EGF induction, cells expressing ARF4 formed membrane ruffles and ARF4 together with EGFR became concentrated in the ruffles. This result suggests that, after EGF induction, ARF4 translocates to membrane ruffles and colocalizes with EGFR.

Next, we examined whether EGF-dependent cell activation would enhance the GTP binding of ARF4. The purified recombinant ARF4 protein was separately incubated with membranes prepared from 293T/EGFR or 293 T cells together with GTP $\gamma$S and treated with or without EGF. The amount of GTP $\gamma$S binding to the ARF4 protein was analyzed by a reconstitution assay as previously described (5). Briefly, cell membranes were prepared from 293/EGFR or 293T cells followed by incubation with EGF and/or recombinant ARF4 in the presence of GTP $\gamma$S. Protein-bound GTP $\gamma$S was separated by filtration through nitrocellulose filters and was quantitated by scintillation counting. In comparison with the GTP $\gamma$S binding to the 293T/EGFR cell membranes alone, no significant enhancement of GTP $\gamma$S binding to cell membranes occurred by incorporating either ARF4 or GTP $\gamma$S proteins in these assays (Fig. 3A). Conversely, treating this membrane preparation in the presence of both ARF4 and EGF notably increased the level of GTP $\gamma$S association with ARF4 (Fig. 3A), indicating that EGF treatment of membranes boosted the binding of GTP $\gamma$S to ARF4. In similar experiments, we used membranes obtained from 293T cells, in which EGFR expression is undetectable. Fig. 3B shows that these ARF4-deficient membranes, there was no increase of GTP $\gamma$S binding to ARF4, neither by adding ARF4 nor by adding ARF4 and EGF. These results demonstrate that EGF enhances the binding of GTP $\gamma$S to ARF4.

We then examined whether the active GTP-bound form of ARF4 proteins attaches to EGFR-enriched membranes and whether the inactive GDP-bound form of ARF4 remains cytosolic. ARF4 with three C-terminal HA tags (ARF4-HA3) was expressed in 293T/EGFR cells. After serum starvation, cells were collected and incubated with GTP $\gamma$S in the presence or absence of EGF. These cells were then treated with digitonin, a detergent that permeabilizes cell membranes and disrupts cytosolic ARF4-HA3 to leak out of the cells. The cytosolic and membrane-containing cell pellet fractions of these cells were separated by SDS-PAGE and analyzed by Western blot with an anti-HA antibody to examine the distribution of ARF4-HA3 between these two fractions. The digitonin-treated cells released most of their ARF4-HA3 into the supernatant, indicat-
ing that most ARF4-HA3 proteins were cytosolic, whereas without digitonin treatment, ARF4-HA3 remained inside the cells (Fig. 3C). In the presence of either GTP·S or EGFR, digitonin-treated cells only partially released ARF4-HA3 into supernatant (Fig. 3C). However, when these cells were treated with EGF in combination with GTP·S, almost all ARF4-HA3 was retained in the cell membrane-containing cell pellet fraction. These data, together with the EGF-induced ARF4 translocation data shown in Fig. 2, indicate that the activation of ARF4 either by EGF or by association with GTP·S induces the translocation of ARF4 from the cytosol to EGFR-enriched cell membranes.

ARF4 Mediates EGF-induced Activation of PLD2—Previous studies have shown that agonist-induced ARF activation and its consequent translocation to cell membranes stimulate PLD activity (13, 15, 16). To investigate whether EGF-induced ARF4 activation is able to stimulate PLD activity in cells, we transiently co-expressed PLD1 or PLD2 together with ARF4 or its mutants in 293T/EGFR cells, followed by EGF stimulation of particular samples. Activation of cellular PLD was determined by the unique ability of PLD to produce, in the presence of butanol, phosphatidylbutanol, which cannot be further metabolized. In cells overexpressing PLD2 and ARF4, PLD activity was dramatically stimulated by EGF treatment (Fig. 4A). However, in cells overexpressing PLD1 and ARF4, this EGF-induced up-regulation of PLD activity was not observed (Fig. 4B). In addition, overexpressing ARF4(Q71L), the dominant active form of ARF4, constitutively stimulated the activity of PLD2 but did not have the same effect on PLD1 (Fig. 4A and B). These data suggest that ARF4 mediates EGF-induced cellular activation of PLD2 but does not mediate activation of PLD1. As a positive control in these experiments, we used the small GTPase RalA, which mediates EGF-induced PLD1 activity and, to a lesser extent, PLD2 activity (17) (Fig. 4A and B).

ARF4 GTPase Regulates Transcriptional Activity of AP-1 through Activation of PLD2—PLD activity, in cooperation with EGFR, has been reported to be involved in the transformation of cells (17). One of the major causes of EGFR-dependent cell transformation is the altered expression of specific target genes that results from changing the activities of certain transcriptional factors by unrestrained signaling effectors. The role of PLD2 in the EGF-mediated modulation of gene expression through transcriptional activation has not been thoroughly examined. We tested whether ARF4-mediated PLD2 activation regulates gene transcription through specific enhancer elements, such as AP-1, interferon GAS, NFAT, or SRE. For these experiments, we used expression plasmids encoding the luciferase gene, driven by a basic promoter element (TATA box) joined to tandem repeats of AP-1, GAS, NFAT, or SRE binding elements. These plasmids were individually transected into 293T/EGFR cells. Expression of activated PLD2 significantly induced luciferase expression driven by the promoter containing AP-1; in contrast, luciferase expression was not induced by promoters containing GAS, NFAT, and SRE binding sites (Fig. 5A). These data suggest that PLD2 activation stimulated by the active form of ARF can enhance AP-1 activity.

We further tested whether this ARF4-mediated PLD2 activation is involved in EGF-induced cellular AP-1 activation. 293T/EGFR cells were transiently transfected with the AP-1- or SRE-driven luciferase reporter constructs and, in some cases, cotransfected with the expression vector encoding ARF4(T31N), followed by EGF treatment (Fig. 5B). EGF-induced AP-1-dependent reporter gene expression more than 3-fold, and this induction of luciferase expression was inhibited considerably by dominant negative ARF4(T31N). In contrast, SRE-dependent reporter gene expression stimulated by EGF was not blocked by ARF4(T31N) (Fig. 5C).

To further confirm and characterize the AP-1 activation by ARF4-mediated PLD2 activation, we used nuclear extracts generated from cells transfected with the combination of expression plasmids as indicated in Fig. 5 for electrophoretic mobility shift assay experiments, and the resultant nuclear extracts were incubated with radiolabeled oligonucleotides containing AP-1 sequences. Binding studies revealed that the AP-1/nuclear protein complex increased dramatically in cells containing activated PLD2 due to the co-expression of the ARF4 active form, ARF4(Q71L) (Fig. 6A). Unlabeled homologous
AP-1 oligonucleotides prevented the binding of the radiolabeled AP-1 sequences to nuclear proteins, whereas mutated AP-1 oligonucleotides did not prevent this binding (Fig. 6A). In addition, to examine the individual components within the AP-1-nuclear protein binding complex, we included antibodies against c-Jun and c-Fos in some of these electrophoretic mobility shift assay experiments (Fig. 6B). We found that these antibodies significantly up-shifted the binding of nuclear proteins from cells containing activated PLD2 to the AP-1 consensus sequence, suggesting the presence of c-Jun and c-Fos in these DNA-protein complexes (Fig. 6B). In 293T/EGFR cells, EGF induces the binding of nuclear proteins to AP-1 (Fig. 6C). We tested whether ARF4 activity is required for EGF-dependent AP-1 activation. We found that the cellular expression of dominant negative forms of ARF4, ARF4(T31N), dramatically reduced this EGF-mediated binding of nuclear proteins to AP-1 oligonucleotides (Fig. 6C), suggesting a critical role of ARF4 activity in EGF-induced AP-1 activation. Taken together, these results suggest an important function of both ARF4 and PLD2 in regulating the transcriptional activity of AP-1 in the EGF-induced cell activation.

Mitogen-activated protein (MAP) kinases have been implicated in the regulation of AP-1 activity (18, 19). To explore the possible involvement of two MAP kinase pathways, BMK1/ERK5 and ERK1/2, in mediating PLD2-induced AP-1 activation, we transfected expression vectors encoding ARF(Q71L) and PLD2 into 293T/EGFR cells and examined the activity of endogenous BMK1/ERK5 and ERK1/2. We found that the activation of PLD2 by ARF(Q71L) had no detectable effect on endogenous BMK1/ERK5 and ERK1/2 activities (Fig. 7). MEK5(D) and MEK1(E), dominant active forms of MEK5 and MEK1, are known to specifically activate BMK1/ERK5 and ERK1/2, respectively. When MEK5(D) or MEK1(E) were expressed in 293T/EGFR cells, the endogenous BMK1/ERK5 or ERK1/2 was activated correspondingly (Fig. 7). These results suggest that these two MAP kinase cascades are not critically involved in AP-1 activation by the ARF4/PLD2 pathway.

**DISCUSSION**

In this study, we have identified ARF4 as an intermediate molecule that interacts with EGFR and have shown that EGF-activated ARF4 protein specifically up-regulates cellular PLD2 but does not up-regulate PLD1 activity. Moreover, we have demonstrated that this ARF4-dependent PLD2 activation significantly enhances the transcriptional activity of AP-1. EGFR is known to cooperate with PLD in cellular transformation, yet the molecular mechanism of this oncogenic process is unknown. One possible route, as indicated by our findings, is via the dramatic enhancement of the activity of the AP-1 oncprotein, resulting from EGF-dependent PLD activation.

ARFs are usually modified by myristoylation at Gly2 through the action of N-myristoyltransferase (20). Nevertheless, Kanoh et al. (21) have shown that a mutant form of ARF3, which is mutated at Gly2 to eliminate myristoylation, does not abolish its interaction with arfaptin 1. In our yeast two-hybrid analysis (Fig. 1A), affinity between the EGFR cytoplasmic domain and ARF4 does not seem to require N-terminal myristoylation. In yeast two-hybrid assay, the ARF4 fusion protein that we used contained an N-terminal GAL4-activating domain, which should have prohibited the ARF4 part of this GAL4-AD/ARF4 fusion protein from being modified by N-myristoyltransferase, an enzyme that can act only at the N-terminal end of a protein (22). Without its N-terminal myristoylation, ARF4 appears to have some affinity with the cytoplasmic domain of EGFR; however, drawing any conclusion is difficult without obtaining additional information about the N-terminal modification of GAL4/ARF4 fusion proteins in yeast. More detailed studies are needed to decipher the contribution of N-terminal myristoylation of ARF4 in its binding to the cytoplasmic domain of activated EGFR.

In general, ARF proteins are involved with intracellular membrane transport within the Golgi complex, between the endoplasmic reticulum and Golgi complex (23), with endosome fusion (24), and with several other processes related to intracellular membrane transport. ARF proteins, with the exception of ARF6, are found...
either dispersed in the cytosol or associated with Golgi membranes (25); ARF6 is permanently bound to plasma membranes. Our discovery indicates that the ARF4 protein is activated by EGFR-mediated cellular activation and is simultaneously translocated from the cytosol to the cellular membrane. Similarly, ARF1 is known to be activated and translocated to the cell membrane by activation of another receptor tyrosine kinase, the insulin receptor (5). Since ARF1 and ARF4 belong to class I and II ARFs, classes that appear to be similar in both their cellular localization and functions, it is not surprising that both ARF1 and ARF4 are activated by receptor tyrosine kinase-mediated cellular activation and translocated to cellular membranes. However, it would be interesting to know whether the other members of class I and II ARFs behave similarly in receptor tyrosine kinase-dependent stimulation of cells.

PLD, consisting of PLD1 and PLD2 isoforms, hydrolyzes phosphatidylcholine to choline and to the second messenger, phosphatidic acid, which can serve as a signal transducer. PLD2 is a 106-kDa protein that shares 50\% homology with PLD1 but lacks the 116-amino acid loop region following the first HKD motif, a highly conserved catalytic motif (H\(\times\)K\(\times\)X\(\times\)D) that is essential for the enzymatic activity of phosphatidylinositol-transferase (26). PLD2 localizes primarily to plasma membranes, in contrast to PLD1, which localizes solely to perinuclear regions (the endoplasmic reticulum, Golgi apparatus, and late endosomes) (27). In comparison with PLD1, the membrane localization of PLD2 makes it a logical mediator for delivering signals generated from receptors that reside in the cellular membrane. Indeed, other than mediating signaling from activated EGFR, as we have demonstrated, PLD2 can also relay intracellular signals descended from activation of the insulin receptor.

AP-1 activity is modulated by a variety of dimers that are composed of members of the Fos, Jun, and ATF families (28).}

**Fig. 6.** EGF-induced ARF4/PLD2 activation enhances the binding of nuclear proteins to AP-1 sequences. 293T/EGFR cells were transfected with different combinations of expression vectors as indicated: none, PLD2, and PLD2 plus ARF4(Q71L). Nuclear extracts prepared from these transfected cells were incubated with [\(\gamma\)-\(32\)P]oligonucleotides. A, incubations were performed in the absence or presence of a 100-fold excess of unlabeled wild-type or mutated oligonucleotides. The arrow indicates the specific DNA-protein complex. B, incubations were performed in the absence (None) or presence of control nonspecific antibodies (NS) or with antibodies specific to the c-Jun (c-Jun (D)) or to c-Fos (c-Fos (K-25)) families of nuclear proteins (Santa Cruz Biotechnology). C, 293T/EGFR cells were transiently transfected with an empty expression vector or vector encoding ARF4(T31N). After transfection, cells were starved overnight followed by stimulation with or without EGF (100 ng/ml) for 10 min. Nuclear extracts were prepared and incubated with labeled oligonucleotides alone or together with a 100-fold excess of unlabeled wild-type or mutated oligonucleotides.

and ARF4 belong to class I and II ARFs, classes that appear to be similar in both their cellular localization and functions, it is not surprising that both ARF1 and ARF4 are activated by receptor tyrosine kinase-mediated cellular activation and translocated to cellular membranes. However, it would be interesting to know whether the other members of class I and II ARFs behave similarly in receptor tyrosine kinase-dependent stimulation of cells.

**Fig. 7.** ARF4-dependent PLD2 activation does not up-regulate activities of BMK1/ERK5 and ERK1/2 MAP kinases. 293T/EGFR cells were transiently transfected with expression vectors encoding ARF4(Q71L), PLD2, MEK5(D), or MEK1(E), and the activity of endogenous BMK1/ERK5 and ERK1/2 was determined after 48 h. The phosphorylation and consequent activation of endogenous BMK1 resulted in a shift in its electrophoretic mobility, detected by Western blot analysis with the anti-BMK1 antibody (top panel) (12). Activation of endogenous ERK1/2 was measured in cell lysates by a phospho-specific anti-ERK1/2 antibody (middle panel) and endogenous ERK1/2 was detected by an anti-ERK1/2 antibody (bottom panel). The transfection efficiency for 293T/EGFR was maintained at 70–80% with the pEGFP-N1 plasmid as described in the legend to Fig. 4, and only plates with this range of efficiency were used.
(29–31). The MAP kinase pathways regulate both the amounts and transactivating capacities of the component Fos, Jun, and ATF proteins of AP-1 in a stimulus-specific manner. For instance, treatment of HeLa cells with platelet-derived growth factor, serum, or phorbol esters predominantly activates the extracellular signal-regulated kinase (ERK) members of the MAP kinase family, leading to strong stimulation of Jun and Fos activity. On the other hand, treatment with stress-inducing stimuli, such as ultraviolet light, activates the c-Jun N-terminal kinase/stress-activated protein kinase members of the MAP kinase family, which preferentially enhance Jun and ATF activity via phosphorylation of c-Jun serines 63 and 73 and ATF2 threonines 69 and 71, which are located in their respective transactivation domains (32). In contrast, activation of protein kinase A by CAMP strongly enhances only de novo synthesis of JunB and c-Fos (33). Here, we show that AP-1 activity is up-regulated by EGF-induced PLD2 activation (Fig. 5). Still, the intermediate signaling pathways by which PLD2 induces the activation of AP-1 are presently unknown. We have explored the possible involvement of two MAP kinase pathways, ERK1/2 and BMK1/ERK5, in mediating PLD2-induced AP-1 activation and have found that the ARF4-dependent activation of PLD2 has no effect on endogenous BMK1/ERK5 and ERK1/2 activities (Fig. 7), indicating that these two kinase cascades of PLD2 has no effect on endogenous BMK1/ERK5 and ERK1/2 activation and have found that the ARF4-dependent activation of BMK1/ERK5, in mediating PLD2-induced AP-1 activity.

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