Phosphatidylinositol-4-phosphate 5-Kinase-1β Is Essential for Epidermal Growth Factor Receptor-mediated Endocytosis*

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M. Alejandro Barbieri‡§, Colin M. Heath‡§, Elizabeth M. Peters‡, Alan Wells‡, J. Nathan Davis†, and Philip D. Stahl***

From the ‡Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110-7463, the §Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130, and the †Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Phosphatidylinositol-4,5-bisphosphate (PIP$_2$) is known to play an important role in signal transduction and membrane trafficking. We show that one enzyme responsible for PIP$_2$ production, phosphatidylinositol-4-phosphate 5-kinase type 1β (PIPKβ), is essential for epidermal growth factor receptor (EGFR)-mediated endocytosis. Expression of murine PIPKβ in NR6 cells expressing EGFR strikingly increased receptor internalization. Moreover, the kinase was shown to form an immunoprecipitable complex with EGFR. Expression of either a truncated kinase or a kinase dead mutant inhibited EGFR endocytosis and also blocked the membrane recruitment of PIPKβ and both clathrin light chain and dynamin. Our results delineate a novel mechanism by which PIPKβ regulates receptor-mediated endocytosis and receptor tyrosine kinase membrane traffic.

Phosphatidylinositols (PIs) are known to play an essential role in membrane trafficking and signal transduction (1). PIs serve multiple functions via the recruitment of cytosolic proteins with PI phosphate (PIP) binding domains (2), the modification of the physical properties of the membranes in which they reside or by serving as a branch point in phosphoinositide metabolism as substrates for phosphoinositide-specific lipases (3). Recent work with the PI phosphatases indicates a central role for PIP$_2$ and PIP$_3$ in receptor-mediated endocytosis, although the precise nature of the PI kinases involved and their role have not been delineated (4, 5).

Signaling by ligand-activated receptor tyrosine kinases (RTKs) triggers the rapid internalization and degradation of receptor-ligand complexes (6, 7), which is the major mechanism for long term signal attenuation (8). Moreover, the intracellular trafficking of receptors may allow for differential signaling according to subcellular locale (9). Thus, receptor internalization impacts the biological responses to growth factor receptors and is a potential target for signal modulation.

EGF receptor autophosphorylation is thought to result in a conformational change in the receptor revealing internalization motifs (10) that allow the receptor to interact, directly or indirectly, with endocytic proteins, such as AP-2, clathrin, and dynamin (10, 11). Interestingly, recent work with colony-stimulating factor-1 receptor (CSF-1R) identified a possible connection between the endocytosis of activated RTKs and the type I phosphatidylinositol-4-phosphate 5-kinase (PIPK) (12). Indeed, PIP$_2$, the product of PIPKs, has been shown to be involved in multiple steps of endocytosis, including the assembly of the clathrin coat, the regulation of adaptor proteins, and the production of endocytic vesicles via the regulation of dynamin (2).

Here, we demonstrate that overexpression of murine phosphatidylinositol-4-phosphate 5-kinase type 1β (PIPKβ) or a truncated construct, PIPKβ-truncated, but not the mouse type 1α isoform (PIPKα), causes dramatic alterations in the endocytic trafficking of the EGFR, including an effect on recruitment of dynamin and clathrin light chain. Our data indicate that PIPKβ plays a key role in mediating the recruitment of the protein machinery required for clathrin-mediated endocytosis.

EXPERIMENTAL PROCEDURES

DNA Construction—The mouse cDNAs of PIPKβ and -β, the N-terminal truncated mutants (Δ1–197α; Δ1–238β)), and the PIPKβ kinase dead mutant (K179M) were subcloned into pCDNA3 or the Sindbis vector Toto1000:3/H9252 (13) without a tag or with a GFP, GST, or HA-tag as indicated. Sindbis virions were generated by using a LipofectAMINE-mediated procedure (Life Technologies, Inc.). Sindbis virus, harvested from spent media 24–40 h after transfection, generally titered between 10$^6$ and 10$^8$ plaque-forming units per ml. Virus stocks were kept frozen at −80 °C before use.

Transfection and Sindbis Virus Infections—NR6 monolayers were either transiently transfected with pcDNA3 vector using Fugene 6 (Roche) as described (12) or mock-infected or infected with the vector or recombinant viruses as described previously (13).

Whole Cell Lysates, Immunoprecipitation, and Western Blotting—NR6 cells that stably express the human EGFR (NR6-EGFR) (8, 14) were serum-starved and incubated with 100 nM EGF for the indicated times at 37 °C. Cell monolayers were washed with phosphate-buffered saline containing 1 mM sodium orthovanadate, 5 mM β-glycerophosphate, and lysed in ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 50 mM Hepes, 100 mM NaCl, 1 mM sodium orthovanadate, 5 mM β-glycerophosphate, 5 mM EDTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, and 2 μg/ml aprotinin, pH 7.2). Lysates were clarified by centrifugation at 16,000 × g for 15 min and the supernatant was used in immunoprecipitation with anti-GFP antibody (Roche). Immunoprecipitates were then washed three times in 1% Nonidet P-40, 10% glycerol, and subjected to Western blot analysis of phosphoproteins using anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Inc.).
Addition of EGF to cells results in the internalization and degradation of EGF (6–8), and therefore we examined the effect of PIPKβ and the two inhibitory mutants on down-regulation of EGFR following exposure to ligand. NR6-EGFR cells were infected with Sindbis virus, as described above, encoding PIPKβ or the inhibitory constructs, and the rate of EGFR down-regulation was examined, as described under “Experimental Procedures.” In control cells, 64% of surface EGFR was lost following exposure to EGF within 3 h. In cells overexpressing PIPKβ, 82% of surface EGFR was lost, whereas the majority of surface EGFR remained at the cell surface in cells expressing either PIPKβ-truncated (24% lost) or PIPKβ-dead (11% lost). Failure to internalize should lead to higher levels of 125I-EGF binding in cells expressing either PIPKβ-truncated or PIPKβ-dead. Indeed, the expression of the two mutants, but not the wild type kinase, substantially increased EGF cell surface binding in transfected cells (data not shown).

We next set out to determine whether PIPKβ-truncated blocked EGF-mediated endocytosis by preventing the recruitment of the cytosolic wild type kinase to the membrane (1). A cell fractionation study (15), using NR6-EGFR cells expressing the PIPKβ or PIPKβ-truncated, revealed that PIPKβ is localized to both membranes and cytosol, whereas PIPKβ-truncated is localized almost exclusively to the cytosolic fraction. Addition of EGF prior to fractionation resulted in a shift in the distribution of the cytosolic wild type kinase to the membrane, whereas PIPKβ-truncated cytosolic distribution was unaffected by EGF (data not shown).

The fact that PIPKβ-truncated is mostly cytosolic and enzymatically inactive (12) raises the possibility that PIPKβ-truncated may interact with the wild type enzyme such that targeting to the plasma membrane is prevented.

To delineate the role of PIPKβ-truncated in inhibiting endocytosis, we examined the effect of expressing PIPKβ-truncated on the targeting of PIPKβ to the plasma membrane in living cells. A transfection/infection protocol, as described in the legend to Fig. 2A, was used to first express PIPKβ and then either GST-PIPKβ-truncated or GFP-PIPKα-truncated. Data in Fig. 2A, panel II, show that GST-PIPKβ-truncated is localized totally to the cytoplasmic compartment. In cells expressing GST-PIPKβ-truncated, PIPKβ is also seen predominantly in the cytoplasm (panel I), suggesting that the truncated construct is blocking the targeting of PIPKβ to the plasma membrane. In control cells (panels I and IV, arrows), PIPKβ is localized to the plasma membrane. A merged image (panel III) shows the co-localization of PIPKβ and GST-PIPKβ-truncated in the cytoplasmic compartment. As an additional control, we used an N-terminal truncation of PIPKα, which is truncated from the same point in the conserved core kinase homology domain (17, 18) as PIPKβ-truncated. The PIPKα isoform has been shown to play a role in membrane ruffling (19–22) and has no effect on EGFR uptake.2 Surprisingly, GFP-PIPKα-truncated localized to the nucleus as shown in panel V, and the merged image (panel VI) shows the distinct localization of PIPKβ and GFP-PIPKα-truncated, indicating that the truncated PIPKα isoform does not block PIPKβ plasma membrane targeting.

To determine whether PIPKβ and PIPKβ-truncated are physically associated, co-immunoprecipitation experiments were carried out using NR6-EGFR cells expressing both GFP-PIPK (a or β) and GST-PIPKβ-truncated. A second set of control experiments were carried out with cells co-expressing HA-

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PIPKβ and GFP-PIPKβ-truncated. Fig. 2B, panels I and II, show Western blots of total lysates indicating the approximate levels of cellular expression of the GFP, GST, and HA constructs. Cell lysates were prepared and immunoprecipitated with anti-GFP antibodies (Fig. 2B, panels III and IV) and following SDS-PAGE, Western blotting with anti-GST antibodies (panel III, upper) indicated that immunoprecipitation of GFP-PIPKβ results in the co-immunoprecipitation of GST-PIPKβ-truncated. On the contrary, GFP-PIPKα failed to co-immunoprecipitate GST-PIPKβ-truncated. As a control (panel IV), immunoprecipitation of GFP-PIPKα-truncated failed to co-immunoprecipitate HA-PIPKα. We speculate that the formation of a complex between the wild type enzyme and truncated construct may interfere with the correct targeting of the kinase to the plasma membrane. This speculation is further supported by gel exclusion chromatography analysis, which suggests that the PIPKβ exists as large multimeric complexes. Previous studies indicate that one or more phosphoinositide kinase activities partially co-purify with EGFR (16) and that EGF stimulates phosphoinositide kinase activity in several cell types (23). To determine whether PIPKβ and PIPKβ-truncated are physically associated with EGFR, cells expressing PIPKβ, PIPKβ-truncated, or both constructs were stimulated with EGF, and immunoprecipitation studies were carried out as described in the legend to Fig. 2C. Fig. 2C, panel I, shows EGFR detected with both anti-EGFR and anti-phosphotyrosine antibodies, and Fig. 2C, panel II, shows a co-immunoprecipitation of PIPKβ with EGFR detected with a PIPKβ-specific antibody. The data also indicate that ligand activation of the receptor results in a significant increase in EGFR-PIPKβ association. On the contrary, EGFR and PIPKβ-truncated failed to co-immunoprecipitate. Moreover, co-expression of PIPKβ-

**Fig. 2.** PIPKβ-truncated, but not PIPKα-truncated, inhibits 125I-EGF uptake by blocking the targeting of PIPKβ to the plasma membrane. A, confocal images of PIPKβ, PIPKβ-truncated and PIPKα-truncated. NR6-EGFR cells were transiently transfected with PIPKβ using a pcDNA3 expression vector for 18 h. After transfection, the cells were infected with Sindbis virus encoding GST-PIPKβ-truncated or GFP-PIPKα-truncated as described in the legend to Fig. 1. After infection, the cells were incubated with 100 nM EGF for 10 min at 37 °C, washed, fixed, and processed for confocal microscopy as described previously (15). Proteins were detected with antibodies specific for PIPKβ (12) (panels I and IV) or polyclonal GST (Sigma) (panel II), and they were visualized with the Alexa antibodies (Molecular Probes) with anti-GFP:PIPKα-truncated was visualized with the intrinsic fluorescence of GFP (panel V). The arrows indicate uninfected cells. Individual confocal sections are shown. Shown is one experiment representative of three independently performed. Bar, 10 μm. B, co-immunoprecipitation of GFP-PIPKβ and GST-PIPKα-truncated. NR6-EGFR cells were transiently transfected with GFP-PIPKβ or GFP-PIPKα using the pcDNA3 expression system; transfected cells were then infected with Sindbis virus encoding GST-PIPKα-truncated. In a second experiment, pcDNA3 was used to express HA-PIPKβ followed by expression of GFP-PIPKα-truncated using the Sindbis expression vector. Transfected/uninfected cells were incubated with 100 nM EGF for 10 min at 37 °C, and after incubation, the cells were washed, lysed, and immunoprecipitated as described previously (12). Equal aliquots of cell lysates were resolved by SDS-PAGE, transferred, and visualized by GFP-(Zymed Laboratories Inc.), GST- (Sigma), or HA- (CLONTECH) specific antibodies as shown in Panels I and II, Panel III, GFP-PIPKα (α and β) were immunoprecipitated using a polyclonal anti-EGFR antibody (CLONTECH), and following SDS-PAGE, GST-PIPKβ-truncated was detected by immunoblotting with monoclonal anti-GST antibody (panel III, upper). The immunoprecipitated GFP-PIPKα and -β were detected with the monoclonal anti-EGFR antibody (Zymed Laboratories Inc.) (panel III, lower). Panel IV, HA-PIPKβ and GFP-PIPKα-truncated were detected by immunoblotting with polyclonal anti-HA (Sigma) and anti-GFP antibodies, respectively (panel IV). The immunoprecipitations were performed with polyclonal anti-GFP antibody followed by immunoblotting with anti-HA antibody and subsequent re-probing with monoclonal anti-GFP antibody. Shown is one experiment representative of three independently performed (12). C, PIPKβ-truncated associates with EGFR, which is blocked by overexpression of PIPKβ-truncated. NR6-EGFR cells were infected with virus encoding either PIPKβ, PIPKβ-truncated, or both constructs as described previously (15). After 1.5 h, the cells were starved for an additional 4 h and then incubated in the presence or absence of 17 nM EGF for 10 min at 37 °C. The cells were lysed, and 150 μg of total protein was used for immunoprecipitations with an antibody specific for the N terminus of EGFR (Abs, Oncogene). Panel I, total EGFR in the assay (upper) and tyrosine-phosphorylated EGFR (lower) was resolved by SDS-PAGE, Western blotting, and probed with antibodies specific for EGFR (Santa Cruz) or phosphotyrosine (Transduction Laboratories), respectively. Panel II, PIPKβ and PIPKβ-truncated associated with EGFR were resolved by SDS-PAGE and Western blotted with an antibody specific for PIPKβ (Upstate Biotechnology). The figures represent relative units (RU) of PIPKβ (upper) or the truncated mutant (lower) associated with the EGFR, with 1 relative unit being the amount of PIPKβ associated with the receptor in the absence of EGF stimulation. A kinase assay (12) was carried out on equivalent immunoprecipitates, and the relative units for the kinase activities were 1, 1.3, 0.14, and 0.22 for the kinase in lanes 1, 2, 5, and 6, respectively. The gel shown is one experiment representative of three independently performed.
PIPKβ truncated with the wild type kinase blocked the co-immunoprecipitation of an EGF-PIPKβ complex. PIPK enzymatic activity, which is regulated by phosphorylation (24), was quantitated in the immunoprecipitates and, as indicated in the legend of Fig. 2C, corresponds with the quantitation of the Western blots (i.e., the lipid kinase which is recruited to EGFR is active). Another factor that may influence the recruitment of PIPKβ is the lipid binding site described by Anderson and colleagues (1), who have suggested that lipid kinase recruitment may include both a lipid binding site and specific protein targeting sites. Our data suggest that the expression of the PIPKβ-truncated inhibits endocytosis of 125I-EGF by blocking the targeting of the PIPKβ to EGFR at the plasma membrane. Interestingly, Tolias et al. (19) have shown that the thrombin activation of PIPKα and β is regulated by Rac, although only PIPKα is involved in Rac-dependent actin assembly. These new data now delineate different functions associated with different isoforms of type 1 PIPKs.

Our data indicate a key role for PIPKβ in the early steps of internalization. PIP2, the product of PIPKβ, has been shown to bind specific domains (25), found in many of the proteins required for endocytosis and signaling. Dynamin and AP2, for example, bind to PIP2 and regulate EGF receptor-mediated endocytosis (26). To investigate whether the expression of PIPKβ, PIPKβ-dead, or PIPKβ-truncated affects the localization of proteins controlling clathrin-coated vesicle formation, cells were transfected with Sindbis virus encoding PIPKβ or the two inhibitory mutants, and the localization of clathrin light chain (CLC) or dynamin, following EGF stimulation, was analyzed by confocal microscopy. In Fig. 3A, we show that the expression of PIPKβ-truncated or PIPKβ-dead blocks the targeting of dynamin to the plasma membrane. GFP-PIPKβ (panel I) is found on the plasma membrane as is dynamin (panel II), where they co-localized (panel III). In addition, dynamin is also localized to intracellular structures. GFP-PIPKβ-truncated localization is cytoplasmic (panel IV), and cells expressing PIPKβ-truncated are unable to recruit dynamin to the plasma membrane (panel V); however, some dynamin in the Golgi region remained (panel V, arrowhead). GFP-PIPKβ-dead localizes to the plasma membrane (panel VI), but dynamin recruitment is still blocked (panel VII), indicating the requirement of an active type 1 kinase for dynamin recruitment in response to EGF.

Recruitment of clathrin light chain is also an index of activated endocytosis, and we examined the effect PIPKβ, PIPKβ-truncated, or PIPKβ-dead on the localization of CLC. Cells stably expressing GFP-clathrin light chain (GFP-CLC) were infected with Sindbis encoding the PIPKβ constructs, stimulated with EGF, and examined by confocal microscopy as described above. In Fig. 3B, panel I, GFP-CLC is localized to punctate spots throughout the cytoplasm. Panel II shows the plasma membrane localization of PIPKβ, and the partial localization of the two proteins in the merged image is seen in panel III. In cells expressing PIPKβ-truncated (panel IV), punctate localization of GFP-CLC is substantially lost, compared with the neighboring untransfected cell (panel IV, arrows), and PIPKβ-truncated is seen localized in the cytoplasm (panel V). Panel VI shows the substantially reduced co-localization of GFP-CLC and PIPKβ-truncated. Last, we examined PIPKβ-dead in cells expressing GFP-CLC. As with the truncated construct, the recruitment of CLC in response to EGF was lost.

Fig. 3. PIPKβ-truncated blocks the association of dynamin and clathrin light chain with the plasma membrane. A, NR6-EGFR cells were transiently transfected for 12 h with pcDNA3 encoding GFP-PIPKβ, GFP-PIPKβ-truncated, or GFP-PIPKβ-dead. After transfection, cells were incubated for 4 additional hours without serum. The cells were then treated with 100 nM EGF for 10 min at 37 °C, washed, fixed, and processed as described in the legend to Fig. 2A, and the localization of dynamin was analyzed by using Alexa594 (red) goat anti-mouse (Molecular Probes) and mouse anti-dynamin antibodies (Transduction Laboratories) (panels II, V, and VIII). The intrinsic fluorescence of GFP was used to visualize GFP-PIPKβ (panel I), GFP-PIPKβ-truncated (panel IV), and GFP-PIPKβ-dead (panel VII). Arrows show untransfected cells, and the arrowhead highlights the Golgi area. Shown is one experiment representative of two independently performed. Bar, 10 μm. B, localization of GFP-clathrin light chain was analyzed in CHO cells stably expressing GFP-CLC. CHO cells were transiently transfected for 12 h with pcDNA3 expressing PIPKβ, PIPKβ-truncated, or PIPKβ-dead. After transfection, cells were incubated for 4 h without serum. The cells were then treated with 100 nM EGF (10 min, 37 °C), washed, fixed, and the localization of PIPKβ was determined using Alexa594 goat anti-rabbit (Molecular Probes) and rabbit anti-PIPKβ antibodies (Upstate Biotechnology). The intrinsic fluorescence of GFP was used to visualize GFP-tagged CLC (panels I, IV, and VII). Arrows show untransfected cells. Shown is one experiment representative of three independently performed. Bar, 10 μm.
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(panel VII). PIPKβ-dead is localized to the plasma membrane (panel VIII), but the absence of co-localization is shown in the merged image (panel IX). These experiments show that recruitment of an active PIPKβ to the plasma membrane in response to EGF is required for the subsequent recruitment of dynamin and clathrin light chains. These observations explain the effect of the truncated kinase on receptor-mediated endocytosis.

Our data indicate that PIPKβ plays a central role in receptor-mediated endocytosis via its recruitment to the EGFR and via the production of its product, PIP2, which in turn affects the binding of specific factors, required for endocytosis. Several groups (19–22) have reported that activation of membrane ruffling is tightly coupled to PIPKα. Thus, it may well be that the various type 1 PIPK isoforms have both independent and possibly sequential functions in signaling leading to endocytosis and ruffling. Expression of PIPKβ-truncated blocks the recruitment of PIPKβ, but additionally, expression of the truncated kinase or the kinase dead mutant appears to block coated vesicle formation by sequestering or preventing recruitment and/or assembly of additional limiting components essential for coated vesicle budding. It is interesting to note that endocytic proteins such as epsin and AP180, which contain ENTH domains, have also been implicated in the endocytosis of the EGFR (27, 28). An additional factor in the regulation of PIPK activity is phospholipase D (PLD). PLD has a required PH domain and is reported to be recruited via the action of PIPKα (29). The product of PLD, phosphatidic acid, is known to be an activator of PIPK activity (30), creating a possible positive feedback scenario for the amplified activation of PIPKβ. Studies in which synaptojanin, a PI(4,5)P2 5-phosphatase, has been deleted confirmed a central role for PIP2 in endocytosis (31). Our studies now directly demonstrate a central and fundamental role for PIPKβ in receptor-mediated endocytosis. However, many additional factors need to be identified, including those factors that are responsible for the recruitment, activation, and deactivation of the kinase.

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