Type 1γ661 Phosphatidylinositol Phosphate Kinase Directly Interacts with AP2 and Regulates Endocytosis*

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The surface expression level of plasma membrane receptors is highly regulated via balanced vesicular trafficking to and from the plasma membrane. An important aspect of this regulation is the internalization of receptors by the clathrin-mediated endocytosis pathway. This process not only internalizes and delivers extracellular ligands to the endosomal transport system, but it also serves to recycle receptors or target them for degradation.

Clathrin-coated vesicles mediate sorting and intracellular transport of membrane-bound proteins. The formation of these coasts is initiated by the assembly of adaptor proteins (AP), which specifically bind to membrane cargo proteins via recognition of endocytic sorting motifs. The lipid signaling molecule phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) is critical for this process, as it serves as both a targeting and regulatory factor. PI(4,5)P2 is synthesized by type I phosphatidylinositol phosphate kinases (PIPKI). We have discovered a direct interaction between the μ2-subunit of the AP2 complex and PIPKIγ661 via a yeast two-hybrid screen. This interaction was confirmed using both the μ2-subunit in glutathione Φ-transferase pulldowns and via coimmunoprecipitation of endogenous PIPKIγ661 with the AP2 complex from HEK293 cells. The interaction is mediated, in vivo, by a tyrosine-based motif in the 26-amino acid tail of PIPKIγ661. Because AP2 regulates endocytosis of transferrin receptor from the plasma membrane, we also examined a role for PIPKIγ661 using a flow cytometry endocytosis assay. We observed that stable expression of wild type PIPKIγ661 in Madin-Darby canine kidney cells enhanced transferrin uptake, whereas stable expression of kinase-dead PIPKIγ661 had an inhibitory effect. Neither condition affected the overall cellular level of PI(4,5)P2. RNA interference-based knockdown of PIPKIγ661 in HeLa cells also had an inhibitory effect on transferrin endocytosis using the same assay system. Collectively, this evidence implies an important role for PIPKIγ661 in the AP2-mediated endocytosis of transferrin.

Assembly of the clathrin coat is a highly orchestrated process that requires the action of several distinct proteins (1), including G-proteins, protein and lipid kinases and phosphatases, and various adaptor proteins, such as the adaptin protein (AP)3 complexes. These complexes specifically bind to integral membrane cargo proteins and serve both as protein docking sites and as nucleation points for assembly of the clathrin lattice. Several AP complexes have been identified, with each complex having a distinct function (2). For example, AP1B is thought to modulate sorting to the basolateral membrane in epithelial cells, whereas AP2 is thought to regulate receptor-mediated endocytosis from the plasma membrane (3–6).

The AP complexes consist of four distinct subunits. The crystal structure of the AP2 complex has recently been solved and consists of two large subunits, α and β2, a medium subunit, μ2, and a small subunit, σ2 (7). The μ2-subunit is critical for the function of the complex as it specifically binds to membrane cargo proteins via tyrosine-based sorting motifs within their cytoplasmic domains (8). This subunit consists of an N-terminal domain, which is buried within the core of the AP2 complex, joined by a flexible linker to a solvent exposed C-terminal domain. In the AP2 crystal, the μ2-subunit C-terminal domain was found to be buried within a pocket formed by the other three subunits of the complex (7). In this “closed” conformation, the μ-subunit is thought to be unable to interact with membrane-bound cargo proteins. The current model for the AP2 cargo binding suggests that the complex shifts into an “open” conformation upon binding to the plasma membrane in the presence of cargo (7, 9). The displaced μ-subunit can then bind to cargo and dock to the membrane.

Assembly of the AP2 complex onto membranes is mediated by a specific phospholipid, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (10). PI(4,5)P2 also regulates numerous proteins that participate in clathrin-mediated endocytosis, including AP180, epsin, and dynamin (11). In addition, PI(4,5)P2 is an important regulatory factor for modulation of the actin cytoskeleton and subsequently vesicular transport (12, 13). Two PI(4,5)P2 binding sites have been identified within the AP2 complex. One binding site is located on the α-subunit and is positioned to dock the AP2 complex to membranes (5, 7). The other binding site is located on the μ2-subunit and formed by a cluster of conserved lysine residues (14). The AP2 structure

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3 The abbreviations used are: AP, adaptor protein; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PIPKI, type I phosphatidylinositol phosphate kinases; MDCK, Madin-Darby canine kidney; RNAi, RNA interference; HA, hemagglutinin; siRNA, small interfering RNA; PBS, phosphate-buffered saline; GST, glutathione Φ-transferase; HPLC, high performance liquid chromatography; WT, wild type; KD, kinase-dead.
suggests that the μ2-subunit binds PI(4,5)P₂ only when AP complex is docked to the membrane and the μ2-subunit is bound to cargo (7, 15). This model has recently been demonstrated using an in vitro reconstituted system coupled with surface plasmon resonance (16). This collection of evidence emphasizes the critical role for PI(4,5)P₂ in both the initial targeting of AP2 to endocytic sites and for stabilizing the μ2-subunit/cargo interaction at the plasma membrane upon transition of the complex into the open conformation.

PI(4,5)P₂ is synthesized at the plasma membrane by type I phosphatidylinositol phosphate kinases (PIPKI). By generating PI(4,5)P₂, PIPKIs are important not only for regulation of vesicular trafficking, but also have functions in cytoskeletal assembly, cell proliferation, apoptosis, signal transduction, and nuclear functions (15, 17, 18). All three of the known PIPKI isoforms (α, β and γ; using the human nomenclature) have been implicated in clathrin-mediated endocytosis. Previous work has shown that expression of PIPKια in NR6 cells resulted in decreased endocytosis of the epidermal growth factor receptor, whereas expression of kinase inactive PIPKιβ inhibited endocytosis (19). Another study found that PIPKιβ expression had a similar effect on transferrin receptor endocytosis in HeLa cells and that RNAi-based knockdown of PIPKιβ expression had an inhibitory effect (20). PIPKιγ has been implicated as the major producer of PI(4,5)P₂ in synaptic nerve terminals and this role has been supported by observed phenotypes in PIPKιγ knock-out mice, where synaptic vesicle endocytosis is generally impaired (21, 22). However, the mechanism by which PIPKIs are specifically targeted to sites of endocytosis for localized generation of PI(4,5)P₂ remains unclear.

The PIPKιγ isomorph is alternatively spliced in cells, resulting in two major variants, PIPKιγ635 and PIPKιγ661, which differ only by a 26-amino acid C-terminal extension. We have discovered a direct interaction between PIPKιγ661 and the μ2-subunit of AP2 via a yeast two-hybrid approach employing the C-terminal 178 amino acids of PIPKιγ661 as bait. Here we confirm that this interaction is direct and occurs in vivo. Using cell lines stably expressing PIPKιγ661 and siRNAs specifically knocking down expression of PIPKιγ, we have also found that this interaction has direct implications on the endocytosis of the transferrin receptor from the plasma membrane. These combined results suggest that PIPKιγ661 may be an important regulatory factor in clathrin-mediated endocytosis.

MATERIALS AND METHODS

Expression Constructs—PIPKιγ661, PIPKιγ635, and PIPKιγ661KD mammalian and bacterial expression vectors were described previously (23, 24). The following constructs were also described previously (24). The following PIPKιγ661 mutants were generated using the QuikChange™ mutagenesis kit (Stratagene) and the following mutagenic primers and their complements: 1yS645F, 5’-GGAGCTGGGTGACTTCCCGGTCTCATAAGC; 1yP646F, 5’-GGAGCTGGGTGACTTCCCGGTCTCATAAGCC; 1yL647V, 5’-GGGTGACTTCCCGGTCTCATAAGCG; 1yP646R, 5’-GCTGGGTGACTTCCCGGTCTCATAAGCG. The 1yY644F mutant was described previously (24). The full-length mouse μ2-subunit yeast two-hybrid clone was obtained from a murine brain cDNA library (Molecular Interaction Facility, University of Wisconsin). A soluble truncation of this subunit was generated by digestion of the μ2-subunit open reading frame with an internal EcoRI site and an external 5’ Xhol site. The resulting fragment was cloned into pET28 and pET42 bacterial expression vectors (EMD Biosciences).

Cell Cultures and Transfection—HEK293, HeLa, and Madin-Darby canine kidney (MDCK) cells were cultured using Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. HEK293 cells were transfected via calcium phosphate with 2 or 5 μg of each expression vector. The cells were used for immunoprecipitation 48 h after transfection.

MDCK Stable Cell Lines—MDCK cells were stably transfected with various PIPKιγ661 constructs, which are under the control of the tetracycline responsive promoter. The transfected MDCK cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1 μg/ml puromycin, and 100 μg/ml hygromycin B to select for stable transfection. The medium was supplemented with 2 μg/ml doxycycline to suppress transgene expression, as doxycycline withdrawal results in expression of transfected PIPKιγ.

Antibodies—Monoclonal mouse anti-human transferrin receptor was purchased from BD Bioscience. Monoclonal anti-α-adaptin (C-8) antibody was obtained from Santa Cruz Biotechnology. Monoclonal mouse anti-HA (16B12) was obtained from Covance and rabbit polyclonal anti-HA antibody was purchased from Upstate. Horseradish peroxidase-conjugated anti-GST antibody was purchased from Amersham Biosciences and horseradish peroxidase-conjugated and monocholonic anti-T7 antibodies were obtained from Novagen. Monoclonal antitulin antibody (8d4) was purchased from Sigma. Monoclonal anti-phosphotyrosine antibody (4G10) was purchased from Upstate. Polyclonal PIPKιγ antiserum was generated and purified as described previously (23). Polyclonal PIPKια antiserum was generated and purified as described previously (25). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories.

Immunoprecipitation and Immunoblotting—Cells were washed twice with ice-cold PBS and subsequently resuspended and lysed in IP buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2% Nonidet P-40, 2 mM Na₃VO₄, 1 mM EDTA, 1 mM EGTA, and 1 mM MgCl₂). Cell lysates were incubated with 50 μl of 1:1 diluted protein A-Sepharose™ and 2 μg of the specified antibody as indicated at 4 °C overnight. The immunocomplexes were separated by 7.5 or 10% SDS-PAGE, and transferred to polyvinylidene difluoride (Millipore Corp.). Chemiluminescent substrate (Pierce) was used for visualization on x-ray film (RPI Corp.).

Protein Expression and Purification in Escherichia coli—Constructs in pET28 or pET42 expression vectors were transformed into BL21(DE3) competent cells (Novagen). Proteins were expressed and purified using His-Bind™ resin following the manufacturer’s instructions (Novagen) or using glutathione-Sepharose™ 4B Fast Flow as per the manufacturer’s instructions (Amersham Biosciences). Tyrosine-phosphorylated recombinant PIPKιγ661 was generated via coexpression with Src and purified as described previously (26).

GST Pulldown Assays—Recombinant T7-tagged PIPKιγ was incubated with GST-μ2 together with glutathione-Sepharose™ 4
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Fast Flow (Amersham Biosciences) in 500 μl of buffer B (PBS, 1% bovine serum albumin, 0.4% Triton X-100, and 2 mM dithiothreitol) for 4 h or overnight at 4°C. The beads were washed with 1 ml of buffer B four times, resolved by SDS-PAGE, and analyzed via Western blot. GST was used as a negative control for nonspecific binding. All other GST pulldowns were performed with the proteins indicated in the same manner.

Transferin Uptake Assays—Stable MDCK cells were grown in 10-cm dishes. Expression of exogenous PIPKIγ was induced for 72 h by withdrawing doxycycline from medium. For transferrin uptake assays, cells were incubated with serum-free medium for 2 h. The serum-starved cells were then incubated with Alexa Fluor 647 transferrin (5 μg/ml, Invitrogen) in binding medium at 37°C for 20 min. After incubation, the cells were washed three times with PBS, three times with ice-cold acid (0.2 M acetic acid and 0.5 M NaCl, pH 4.1), and three times with PBS again. Cells were finally collected by trypsinization and washed once with PBS. Half of the cells were used to determine PIPKIγ expression by flow cytometry. Briefly, cells were first incubated with primary anti-HA antibody and then with fluorescein isothiocyanate-labeled secondary antibody. The fluorescence intensities of fluorescein isothiocyanate staining were detected from ~10,000 cells and used to determine PIPKIγ expression. The other half of the cells was suspended in 0.5 ml of PBS for determination of transferrin uptake using a FACS CaliburTM (BD Biosciences) flow cytometer. Fluorescence intensities of internalized Alexa Fluor 647 transferrin were quantified from ~10,000 cells.

RNA Interference—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The cells were passed into 60-mm dishes 1 day prior to transfection. The cells were transfected with a PIPKIγ-specific siRNA oligo (5’-AAGGACCUGGACUUCAUGCG) using OligofectamineTM (Invitrogen) transfection reagent. Scrambled control siRNA (5’-AAGACUGCUACAGCUGCG) or PIPKIα-specific siRNA (5’-AAGAUUGGAGCATGACCUG) and PIPKIκ-specific siRNA (5’-AAGAGUGGAGCAUCUCUGG) were used as controls. After 24 h, the cells were transfected again in the same manner. The cells were then used for transferrin uptake assays 72 h post-transfection.

Metabolic Labeling and Determination of Cellular P(4,5)P2 Levels—MDCK cells were metabolically labeled with 20 μCi/ml [3H]inositol (PerkinElmer Life Sciences) and the lipids were extracted and deacylated as described previously (27). The deacylated glycerophosphoinositol phosphates were resuspended in water prior to analysis by HPLC. The deacylated lipids were separated using a Zorbax SAX column and a gradient of 1.3 M ammonium phosphate (pH 3.85). The level of cellular P(4,5)P2 was determined with a Packard in-line liquid scintillation flow detector using deacylated [3H]P(4,5)P2 (PerkinElmer Life Sciences) as a standard.

Immunofluorescence and Microscopy—Cells were washed with PBS at room temperature, fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, and permeabilized with 0.2% Triton X-100 in PBS at room temperature for 10 min. The cells were blocked with 3% bovine serum albumin in PBS at room temperature for 1 h, incubated with the primary antibody for 1 h at 37°C, and washed with 0.1% Triton X-100 in PBS. The cells were then incubated with fluorescent-labeled secondary antibody at room temperature for 45 min and washed with 0.1% Triton X-100 in PBS. The coverslips were mounted to glass slides in Vectashield (Vector Laboratories) mounting medium. Fluorescent images were captured using a Nikon Eclipse TE2000-U microscope with a CoolSNAP CCD camera (RS Photometrics) or using a ×60 Plan oil immersion lens on a confocal laser-scanning microscope (model MR-1000; Bio-Rad) mounted transversely to an inverted microscope (Diaphot 200, Nikon; W. M. Keck Laboratory for Biological Imaging, Madison, WI). Images were processed as described previously using PhotoshopTM CS (23). To visualize the colocalization between AP2 and PIPKIγ661 constructs, MDCK cells in 6-well plates were transfected with 1 μg of each expression vector. After 16 h of expression, the cells were incubated in serum-free Dulbecco’s modified Eagle’s medium for 2 h and then treated with 50 μg/ml of transferrin (Invitrogen) for 20 min at 37°C. The cells were subsequently fixed and stained as described above.

RESULTS

PIPKIγ661 Directly Interacts with the μ-Subunit of AP2 in Vitro and in Vivo—Our laboratory performed a yeast-two-hybrid screen using the C-terminal 178 amino acids of PIPKIγ661 as bait (23). This screen indicated a direct association with the μ2-subunit of AP2. The prey, obtained from a murine brain library, contained the entire mouse μ2-subunit with the exception of the start codon. To confirm that this interaction was indeed direct, a GST pulldown approach was used.

Because the full-length μ2-subunit is primarily insoluble in E. coli, a truncation mutant was generated. This was accomplished by deleting of the bulk of the N-terminal domain and replacing it with GST. The resulting soluble construct contained the complete linker domain and the C-terminal domain. The GST pulldown was then performed by incubating PIPKIγ661 with GST-μ2 in the presence of glutathione-conjugated SepharoseTM beads. As shown in Fig. 1A, PIPKIγ661 directly associated with GST-μ2, but did not associate with GST alone.

In vivo, AP2 serves as an adaptor for binding to several other proteins involved in clathrin-mediated endocytosis. Consequently, to ensure that this interaction was relevant in vivo, we immunoprecipitated endogenous AP2 from HEK293 cells using a monoclonal antibody specific for α-adaptin. The precipitated complexes were washed extensively and resolved by SDS-PAGE. Using a polyclonal antibody described previously, PIPKIγ was detected in the α-adaptin lane but not in the normal mouse IgG lane (Fig. 1B) (23). The reciprocal experiment, using the monoclonal antibody specific for α-adaptin, yielded concurrent results (Fig. 1B). It was surprising to observe that only the highest molecular weight band was retained by immunoprecipitated AP2. This band corresponds to PIPKIγ661, as the PIPKIγ polyclonal antibody detects both PIPKIγ661 and PIPKIγ661 splice variants, as seen in the lysate lane.

Because P(4,5)P2 has extensively been shown to be an integral component of the endocytic process, we sought to determine whether PIP kinase activity was necessary for this interaction in vivo. Wild type and kinase inactive PIPKIγ661 were transfected into HEK293 cells via calcium phosphate. AP2 was then immunoprecipitated using monoclonal antibodies specific for the α-subunit. As shown in Fig. 1C, both wild type...
A coimmunoprecipitation approach was used to confirm which of the two splice variants could associate with AP2 in vivo. HEK293 cells were transfected with PIPKγ661 or PIPKγ635 and endogenous AP2 was immunoprecipitated with the α-adaptin-specific antibody. As seen in Fig. 1D, only PIPKγ661 was capable of binding to AP2 in vivo. This result indicated that the in vivo binding site for μ2 was localized in the C-terminal 26 residues of PIPKγ661. To narrow the specific binding site, three previously generated truncations of PIPKγ661 were employed in the same coimmunoprecipitation approach (24). As shown in Fig. 1D, truncation at Trp<sup>642</sup> resulted in reduction of associated PIPKγ to background levels observed in the normal mouse IgG control.

**A Tetrapeptide Motif on PIPKγ661 Mediates the Interaction with AP2**—Upon closer inspection of the sequence contained between the Trp<sup>642</sup> and Tyr<sup>649</sup> truncations, there is a putative tyrosine sorting motif (644YSPL647). These motifs generally consist of a tetrapeptide sorting sequence YXXΦ, where Φ corresponds to a bulky hydrophobic residue (8). Through an exhaustive peptide library screen, Ohno et al. (28, 29) has presented extensive evidence documenting the tyrosine sorting motif specificity for each of the μ-subunits of the AP complex. Using these results as a guide, several point mutations were generated with the intention of weakening PIPKγ661 binding to AP2. A mutation intended to strengthen binding was also generated as a positive control. Each of these HA-tagged constructs was transfected into HEK293 cells and endogenous AP2 was immunoprecipitated with a α-adaptin-specific antibody. Associated PIPKγ661 was then detected by immunoblot with an HA-specific monoclonal antibody.

The observed results, shown in Fig. 2A, followed the affinities predicted by Ohno et al. (28). Mutation of Tyr<sup>644</sup>, Pro<sup>646</sup> (Tyr<sup>+2</sup>), or Leu<sup>647</sup> (Tyr<sup>+3</sup>) to the most disfavored residues resulted in disruption of the interaction. The Tyr<sup>+1</sup> position was previously shown to have little contribution to binding affinity in the Ohno et al. (28) peptide screen, and mutation of Ser<sup>645</sup> to the least favored residue, phenylalanine, had little effect on AP2 binding. Likewise, mutation of Pro<sup>646</sup> (Tyr<sup>+3</sup>) to the most favored residue, arginine, did not alter PIPKγ661 binding to AP2. These in vivo results were confirmed via GST pulldown experiments. As in Fig. 1A, GST-μ2 was used to pull down PIPKγ661, PIPKγ635, PIPKγ<sub>644</sub>F, or PIPKγ<sub>647</sub>V. The incubation buffer was supplemented with 1% bovine serum albumin to inhibit nonspecific interactions. As shown in Fig. 2B, PIPKγ661 was specifically retained by GST-μ2, whereas none of the PIPKγ constructs were associated with GST alone. These combined results suggest that PIPKγ661 contains a tyrosine sorting motif that is recognized by the μ2-subunit of AP2 and mediates the direct interaction between these two proteins.

Previous work performed by Ohno et al. (28) has also demonstrated that phosphorylation of the tyrosine residue within YXXΦ sorting motifs disrupts the association of such motifs with the μ2-subunit. We have previously shown that Tyr<sup>644</sup> of PIPKγ661 is phosphorylated by Src in a focal adhesion kinase-dependent manner (23, 24). Consequently, tyrosine phosphorylation of this residue might disrupt the association between PIPKγ661 and the μ2-subunit. To address this possibility,
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we performed in vitro GST pulldowns by incubating GST-μ2 with PIPKIγ661 or tyrosine-phosphorylated recombinant PIPKIγ661, generated as described previously (26). As shown in Fig. 2C, tyrosine phosphorylated PIPKIγ661 associated with much lower affinity as compared with nonphosphorylated PIPKIγ661. This result is consistent with the requirement of an unphosphorylated tyrosine within the YXXφ sorting motif and also might serve as a regulatory mechanism for the interaction between AP2 and PIPKIγ661.

PIPKIγ661 and AP2 Partially Colocalize in MDCK Cells—Because we had determined that PIPKIγ661 interacts with the AP2 complex both in vivo and in vitro, we next examined whether PIPKIγ shared a similar subcellular localization with AP2. Endogenous PIPKIγ and AP2 were immunostained with antibodies specific for PIPKIγ and α-adaptin, respectively, in MDCK cells and examined by confocal microscopy. As shown in Fig. 3A, PIPKIγ is primarily targeted to the plasma membrane and to sites of cell–cell contacts in MDCK cells. This localization also overlaps with the punctuate plasma membrane staining of endogenous AP2, and can be observed not only at the plasma membrane, but also at discrete locations within the cytoplasm (as indicated by the arrows in the merged images). This partially overlapping staining at the plasma membrane was not surprising, as PIPKIγ661 has been shown to serve several roles at the plasma membrane, including regulation of focal adhesion assembly via direct interaction with talin (23, 30). It is important to note that the PIPKIγ polyclonal antibody employed here detects multiple PIPKIγ splice variants expressed in MDCK cells, and we have shown that the interaction with AP2 is specific for only the PIPKIγ661 splice variant.

To further examine the specificity of the interaction between PIPKIγ661 and AP2, HA-tagged WT PIPKIγ661, T644F, and PIPKIγ661 S645F constructs were expressed in MDCK cells. Expressed under normal conditions, all three HA-PIPKIγ661 constructs were targeted to the plasma membrane in a similar manner (data not shown). However, upon stimulation of clathrin-mediated endocytosis via treatment with transferrin, distinct colocalization patterns were observed. As shown in Fig. 3B, both WT and S645F PIPKIγ661 colocalized with AP2 in internalized vesicular structures upon treatment with transferrin. However, in cells expressing PIPKIγ661 T644F, the HA-PIPKIγ signal remained at the plasma membrane and was not significantly internalized under identical conditions. These data collectively support both the specificity of the PIPKIγ661/AP2 interaction demonstrated in vivo and in vitro and also reinforce the functional implications observed in transferrin uptake experiments described below.

The interaction between PIPKIγ661 and AP2 may be a transient event, occurring primarily to facilitate targeting of PIPKIγ661 to sites of endocytosis for localized generation of PI(4,5)P₂. This dynamic association would be necessary for recognition of cargo proteins by the μ2-subunit upon assembly of the AP2 complex onto the plasma membrane, because PIPKIγ661 would occupy the cargo binding site when directly associated with AP2. Consequently, the vesicular cytoplasmic colocalization patterns observed in Fig. 3, A and B, may be PIPKIγ661 directly associated with AP2 during the cycling of the endocytic machinery.

PIPKIγ661 Regulates AP-2-dependent Transferrin Endocytosis—There is considerable evidence that endocytosis from the plasmamembranemediatedbytheAP2complexisPI(4,5)P₂-dependent process (11, 31). The observed endogenous colocalization and the direct interaction between the μ-subunit of AP2 and PIPKIγ661 collectively imply that this interaction may have implications on AP2 function. To address this possibility we generated stable MDCK cell lines inductively expressing either wild type or kinase-dead PIPKIγ661. PIPK expression was
induced by withdrawal of doxycycline from the growth media. After 72 h of expression, the cells were subjected to an endocytosis assay via treatment with Alexa Fluor 647 transferrin and the amount of internalized transferrin was then assessed via flow cytometry. The results from these assays, shown in Fig. 4, A and B, demonstrate that stable expression of wild type PIPKIγ661 resulted in an over 40% average increase of mean fluorescence intensity relative to nonexpressing cells. The opposite effect was observed in cells expressing kinase-dead PIPKIγ635, with a 25% average decrease of intensity.

To confirm that these effects on transferrin endocytosis were due to a specific interaction with PIPKIγ661, we also generated MDCK stable cell lines inducibly expressing wild type or kinase-dead PIPKIγ635. As shown in Fig. 4B, induced expression of either wild type or kinase-dead PIPKIγ635, under the same conditions, had no appreciable effect on transferrin endocytosis. In addition, similar results were also obtained using an MDCK stable cell line inducibly expressing PIPKIγ661 with a T644F mutation (data not shown). These results were consistent with both our in vitro and in vivo interaction data, which indicated that only the PIPKIγ661 splice variant containing the YSPL motif is capable of direct interaction with the μ-subunit of the AP2 complex.

Previous work by Morgan et al. (32) has also proposed a link between trafficking of the AP2 complex and the focal adhesion protein talin. Additionally, the established binding site for talin on PIPKIγ661 does overlap with the YSPL motif necessary for the direct interaction with AP2 (23, 30). To uncouple these two distinct interactions for PIPKIγ661, we generated a stable cell line expressing the S645F mutant. As shown in Fig. 2A, this mutation does not affect binding to the AP2 complex in vivo; however, this mutation does disrupt the interaction between PIPKIγ661 and talin as observed by coimmunoprecipita-
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**FIGURE 4.** Increased PIPKIγ661 expression modulates transferrin receptor endocytosis. A, MDCK cells stably expressing PIPKIγ661 wild type or kinase dead (KD) under the control of the tetracycline repressor were used in a transferrin (Tfn) uptake assay as described under "Materials and Methods." Withdrawal of doxycycline (DOX) results in expression of PIPKIγ661. The histograms for both transferrin uptake and the level of induced protein, as detected using an HA monoclonal antibody, are shown (+DOX, filled peak; −DOX, unfilled peak). B, the mean fluorescence intensity of cells expressing each of the indicated PIPKIγ constructs were normalized to the mean observed for cells with expression repressed by DOX and the average change in transferrin uptake is shown from three independent experiments. Error is shown as 1 S.D. C, HEK293 cells were transfected with HA-tagged wild type or S645F PIPKIγ661 (S645F) as described under "Materials and Methods." Reciprocal immunoprecipitations (IP) were performed using anti-HA, anti-talin, and normal mouse IgG (mIgG) antibodies. The precipitated complexes were resolved by SDS-PAGE and blotted as indicated. D, MDCK cells were transfected with wild type PIPKIγ661, PIPKIγ661 Y644F (Y644F), or PIPKIγ661 S645F (S645F) as in C. Talin and PIPKIγ were visualized by confocal microscopy using antibodies specific for talin (green) and PIPKIγ (red). A merge of the two channels is shown to the right. Scale bar, 10 μm.

PIPKIγ661 Expression Does Not Alter Cellular PI(4,5)P₂ Levels in MDCK Cells—Because potential changes in PI(4,5)P₂ levels alone might be attributed to the observed effects, we quantified total cellular PI(4,5)P₂ levels via metabolic labeling and HPLC. The PIPKIγ661 WT and KD stable MDCK cell lines were cultured in the presence of myo-[³²P]inositol and protein expression was induced for the same duration as in the transferrin uptake assays. The cellular lipids were extracted, deacylated, and resolved by anion exchange. The PI(4,5)P₂ peak was identified using a deacylated PI(4,5)P₂ standard and quantified by total counts using an in-line flow scintillation counter (Fig. 5C). Induction of expression was verified by immunoblot of unlabeled cells (Fig. 5B). As seen in Fig. 5A, expression of either PIPKIγ661 WT or PIPKIγ661 KD did not have a significant effect on the total cellular PI(4,5)P₂ level. Because the total PI(4,5)P₂ levels are not significantly affected by increased PIPKIγ661 WT or PIPKIγ661 KD expression, it is likely a modulation of highly localized pools of PI(4,5)P₂ is responsible for the observed effects on transferrin endocytosis. The relatively stable level of cellular PI(4,5)P₂ is also consistent with previously reported observations for increased expression of PIPKIγ661 in other cell lines (34).

Transferrin Endocytosis Is Inhibited as a Result of Reduced PIPKIγ Expression—An RNAi based approach was also employed as an alternative method for addressing a possible role for PIPKIγ661 in transferrin receptor endocytosis. Using an siRNA oligo specific for the human PIPKIγ, we knocked down PIPKIγ expression in HeLa cells. These cells were then used in
was inhibited on average by 50% in cells transfected with PIPKIγ siRNA. However, no significant effect was observed with either nonspecific control siRNA or with siRNA specific for PIPKIα. The results observed for knockdown of PIPKIα via siRNA was also consistent with results reported previously in HeLa cells (34). The level of knockdown for each protein via RNAi is shown in Fig. 6C.

A change in the expression level of the transferrin receptor could also contribute to the observed inhibition of transferrin endocytosis upon knockdown of PIPKIγ. To rule out this possibility, we assessed the transferrin receptor expression level under these conditions by Western blot. As shown in Fig. 6D, knockdown of PIPKIγ661 does not affect the overall expression level of the transferrin receptor, implying that the observed effects on endocytosis may be a direct consequence of reduced expression of PIPKIγ.

**DISCUSSION**

PI(4,5)P₂ is well established as a major regulatory factor for clathrin-mediated endocytosis (11). Not only does it serve to directly modulate the activity of several proteins necessary for clathrin coat assembly and vesicle formation, but it also functions as an important targeting molecule. The AP2 complex is an excellent example, as it contains two documented PI(4,5)P₂ binding sites, with one site located on the α-subunit and the other on the μ2-subunit (7, 14). However, the specific mechanism responsible for targeted generation of PI(4,5)P₂ at sites of endocytosis remains unclear.

Here we have shown that PIPKIγ661 directly interacts with the μ2-subunit of the AP2 adaptor complex both in vitro and in vivo. This direct interaction is dependent on a tetrapeptide sequence in the PIPKIγ661 C terminus that conforms to a YXXd consensus sorting motif. We have shown that mutation of any of the key residues within this motif results in disruption of the interaction with the μ2-subunit both in vivo and in vitro. The direct interaction between AP2 and PIPKIγ661 provides a mechanism for targeting PIPKIγ661 to sites of endocytosis at the plasma membrane (Fig. 7). Consequently, this would result in generation of a highly concentrated pool of PI(4,5)P₂ at these sites.

The structure for the AP2 core provides some insight to a possible mechanism for regulation of this interaction. In the AP2 crystal, the μ2-subunit is buried in a groove formed by the α- and β2-subunits (7). In this closed conformation, the μ2-subunit YXXd docking site is positioned away from the membrane docking site of the complex. It has been proposed that phosphorylation of the linker domain of μ2 might trigger a conformational change that would allow the subunit to swing out of the pocket into an open conformation and bind to cargo motifs at the plasma membrane (7, 35–37). This structural shift allows for enhanced AP2 membrane association via a direct interaction between μ2 and PI(4,5)P₂, which is not possible in the nonphosphorylated, closed conformation (16). Because PIPKIγ661 binding would occupy the cargo binding site, PIPKIγ661 may bind to AP2 in this inactive conformation.

Upon docking to the plasma membrane, PIPKIγ661 could be displaced from μ2 by either a conformational change in μ2 or by competition of sorting motifs of higher affinity. This dis-

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**FIGURE 5. Cellular PIP₂ levels are not altered by PIPKIγ661 expression.** A, MDCK inducible expressing wild type or kinase-dead PIPKIγ661 were induced or repressed for expression and labeled with myo-[³H]inositol (20 μCi/ml) for 72 h. The cells were then washed and the lipids extracted and decayed as described under “Materials and Methods.” The triplicate results for total counts were normalized against the average total counts in the absence of PIPKIγ661 induction (+ Dox). Data are representative of at least 3 independent experiments. Error is shown as 1 S.D. B, immunoblot demonstrating the stable induction levels of PIPKIγ661 WT and KD in the MDCK cells. The cells were grown as in C in the absence of myo-[³H]inositol. The cells were dissolved in sample buffer, resolved by SDS-PAGE, and immunoblotted as indicated. C, typical HPLC chromatograms for individual samples of PIPKIγ661 WT MDCK stable cell lines + doxycycline or − doxycycline. Relative retention times are indicated for phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI₄P), and PI(4,5)P₂.

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the same transferrin uptake assay as utilized with the MDCK PIPKIγ661 stable cell lines. A nonspecific siRNA oligo was used as a control for normalizing transferrin uptake. As an additional control, we knocked down PIPKIα with an siRNA oligo specific for this isoform. As shown in Fig. 6, A and B, transferrin uptake
FIGURE 6. Decreased PIPKιγ661 expression inhibits transferrin receptor endocytosis. A, HeLa cells were transfected with control siRNA, siRNA specific for PIPKιγ, or siRNA specific for PIPKια. These cells were then used in transferrin uptake assays and fluorescence intensity was quantified as described in the legend to Fig. 5A. The following colors correspond to each RNAi treatment: solid black, control siRNA; solid gray, PIPKια siRNA; and broken gray, PIPKιγ siRNA. Untransfected control cells not treated with transferrin are shown as a filled peak. B, the level of transferrin uptake was measured by the difference in the mean fluorescence obtained from ~10,000 cells by the fluorescence-activated cell sorting assay as described in the legend to Fig. S. The mean intensities of PIPKιγ and PIPKια siRNA-treated cells from three independent experiments were normalized to the mean fluorescence observed in cells treated with control siRNA for each experiment. Error is shown as 1 S.D. C, immunoblot demonstrating the level of knockdown mediated by each siRNA. Cell lysates were resolved by SDS-PAGE and immunoblotted (IB) as indicated. D, HeLa cells were transfected with control or siRNA oligos specific for PIPKιγ as in A. The cells were then lysed with sample buffer, resolved by SDS-PAGE, and immunoblotted as indicated.

placement could also be facilitated by tyrosine phosphorylation of PIPKιγ661. Ohno et al. (29) has previously demonstrated that phosphorylation of the tyrosine within the YXXΦ motif inhibits the interaction with μ2 in cargo peptide binding studies (29). We have previously demonstrated that Tyr644 of PIPKιγ661 is preferentially phosphorylated by Src (24). We have also demonstrated that tyrosine phosphorylation of PIPKιγ661 disrupts the association with the μ2-subunit in vitro. Additionally, several tyrosine kinase receptors trigger Src activation upon binding to extracellular ligands (38). Consequently, PIPKιγ661 would likely become phosphorylated and dissociate from AP2 upon targeting to activated tyrosine kinase receptors or to sites where Src may be active. Therefore, phosphorylation of Tyr644 on PIPKιγ661 could potentially serve as an important regulatory mechanism for the interaction between PIPKιγ661 and AP2 at the plasma membrane.

This putative mechanism for targeting of PIPKιγ661 to endocytic sites is highly reminiscent of the model previously proposed by our laboratory for targeting of PIPKιγ661 to focal adhesions. Upon targeting to focal adhesions via direct interaction with talin, PIPKιγ661 may be competitively displaced from talin by β1-integrin (24). Thus, both models involve a mechanism where PIPKιγ661 is delivered to the plasma membrane and then competitively displaced from its binding partner, while concurrently generating a highly localized pool of PI(4,5)P2 in a tightly regulated fashion.

Additional targeting factors may also recruit PIPKιγ661 to endocytic sites in a concerted manner. The small G-protein ADP-ribosylation factor 6 has previously been shown to enhance PIPKιγ661 activity at endocytic sites and thereby stimulate recruitment of AP2 to the plasma membrane (39). ADP-ribosylation factor 6 has also been shown to associate with the AP2 complex in coimmunoprecipitation experiments (40). PIPKιγ661 might serve as the bridging factor linking ADP-ribosylation factor 6 to the AP2 complex, thereby promoting AP2 assembly via enhanced PI(4,5)P2 production by PIPKιγ661.

It is not surprising that several previous studies have linked various type 1 PIPK isoforms to clathrin-mediated endocytosis. Barbiere et al. (19) has demonstrated in a previous study that expression of wild type PIPKια enhanced endocytosis of epidermal growth factor receptor, whereas expression of a kinase-dead mutant had an inhibitory effect in NR6 cells. Alternatively, Padron et al. (20) recently reported that either expression or RNAi-based knockdown of PIPKιβ had a significant impact on transferrin endocytosis, whereas expression or knockdown of PIPKια or PIPKιγ had no effect in CV-1 and HeLa cells. The authors concluded, however,
that these observed effects were the result of large perturbations of cellular PI(4,5)P₂ levels upon modulation of PIPKIβ expression.

Our results for the effects of PIPKIα knockdown were consistent with this study, as we also observed no significant change in transferrin endocytosis under these conditions in HeLa cells. We also observed no change in PI(4,5)P₂ levels in MDCK cells when stably expressing either wild type or kinase-dead PIPKIγ661 or PIPKIα (data not shown). However, our results directly conflict with the results reported by Padron et al. (20), both for the endocytosis of transferrin receptor and cellular PI(4,5)P₂ levels. This might be attributed to the difference in the cell lines used (CV-1 versus MDCK) or the fact that our methods utilize stable cell lines, whereas Pardon et al. (20) used a transient transfection method for assessing a role for PIPKIγ in transferrin receptor endocytosis. High overexpression levels of PIPKIIs obtained via transient transfection often cause a nonspecific accumulation of PI(4,5)P₂ in the cytosol, resulting in the cells rounding-up or even cell death, which complicates delineation of the phenotypic effects mediated by expression of the PIPKI. Still, our results imply that the direct interaction between AP2 and PIPKIγ661 likely results in modulation of highly localized pools of PI(4,5)P₂, rather than significant alterations of the overall cellular level of PI(4,5)P₂ in the context of elevated or knockdown protein expression levels.

Additionally, we have discovered that the µ1β subunit of AP1B directly interacts with PIPKIγ661 and this subunit binds specifically to PIPKIγ661 in vitro (data not shown). The µ1β- and µ2-subunits both share similar affinities for YXXφ sorting motifs, and this has been documented previously (28).

PIPKIγ661 in endocytosis may be cell-line or tissue specific. Still, the direct interaction between AP2 and PIPKIγ661 provides an attractive mechanism for spatial and temporal generation of PI(4,5)P₂ at sites of AP2-mediated endocytosis. Further examination of precisely how this interaction is modulated will lead to a greater understanding of how clathrin-mediated endocytosis is regulated in cells.

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REFERENCES

1. Brodsky, F. M., Chen, C. Y., Knuehl, C., Towler, M. C., and Wakeham, D. E. (2001) Annu. Rev. Cell Dev. Biol. 17, 517–568
2. Robinson, M. S., and Bonifacino, J. S. (2001) Curr. Opin. Cell Biol. 13, 444–453
3. Folsch, H., Ohno, H., Bonifacino, J. S., and Mellman, I. (1999) Cell 99, 189–198
4. Gan, Y., McGraw, T. E., and Rodriguez-Boulan, E. (2002) Nat. Cell Biol. 4, 605–609
5. Kirchhausen, T. (1999) Annu. Rev. Cell Dev. Biol. 15, 705–732
6. Folsch, H., Pyapaert, M., Schu, P., and Mellman, I. (2001) J. Cell Biol. 152, 595–606
7. Collins, B. M., McCoy, A. J., Kent, H. M., Evans, P. R., and Owen, D. J. (2002) Cell 109, 523–535
8. Bonifacino, J. S., and Traub, L. M. (2003) Annu. Rev. Biochem. 72, 395–447
9. Kirchhausen, T. (2002) Cell 109, 413–416
10. Gaidarov, I., and Keen, J. H. (1999) J. Cell Biol. 146, 755–764
11. Martin, T. F. (2001) Curr. Opin. Cell Biol. 13, 493–499
12. Qualmann, B., Kessels, M. M., and Kelly, R. B. (2000) J. Cell Biol. 150, F111–116
13. Qualmann, B., and Kelly, R. B. (2000) J. Cell Biol. 148, 1047–1062
14. Rohde, G., Wenzel, D., and Haucke, V. (2002) *J. Cell Biol.* **158**, 209–214
15. Martin, T. F. (1998) *Annu. Rev. Cell Dev. Biol.* **14**, 231–264
16. Honing, S., Ricotta, D., Krauss, M., Spate, K., Spolaore, B., Motley, A., Robinson, M., Robinson, C., Haucke, V., and Owen, D. J. (2005) *Mol. Cell* **18**, 519–531
17. D’Santos, C. S., Clarke, J. H., and Divecha, N. (1998) *Biochim. Biophys. Acta* **1436**, 201–232
18. Toker, A. (1998) *Curr. Opin. Cell Biol.* **10**, 254–261
19. Barbieri, M. A., Heath, C. M., Peters, E. M., Wells, A., Davis, J. N., and Stahl, P. D. (2001) *J. Biol. Chem.* **276**, 47212–47216
20. Padron, D., Wang, Y. J., Yamamoto, M., Yin, H., and Roth, M. G. (2003) *J. Cell Biol.* **162**, 693–701
21. Wenk, M. R., Pellegrini, L., Klenchin, V. A., Di Paolo, G., Chang, S., Daniell, L., Arioka, M., Martin, T. F., and De Camilli, P. (2001) *Neuron* **32**, 79–88
22. Di Paolo, G., Moskowitz, H. S., Gipson, K., Wenk, M. R., Voronov, S., Obayashi, M., Flavell, R., Fitzsimonds, R. M., Ryan, T. A., and De Camilli, P. (2004) *Nature* **431**, 415–422
23. Ling, K., Doughman, R. L., Firestone, A. J., Bunce, M. W., and Anderson, R. A. (2002) *Nature* **420**, 89–93
24. Ling, K., Doughman, R. L., Iyer, V. V., Firestone, A. J., Bairstow, S. F., Mosher, D. F., Schaller, M. D., and Anderson, R. A. (2003) *J. Cell Biol.* **163**, 1339–1349
25. Zhang, X., Loijens, J. C., Boronenkov, I. V., Parker, G. J., Norris, F. A., Chen, J., Thum, O., Prestwich, G. D., Majerus, P. W., and Anderson, R. A. (1997) *J. Biol. Chem.* **272**, 17756–17761
26. Bairstow, S. F., Ling, K., and Anderson, R. A. (2005) *J. Biol. Chem.* **280**, 23884–23891
27. Serunian, L. A., Auger, K. R., and Cantley, L. C. (1991) *Methods Enzymol.* **198**, 78–87
28. Ohno, H., Aguilar, R. C., Yeh, D., Taura, D., Saito, T., and Bonifacino, J. S. (1998) *J. Biol. Chem.* **273**, 25915–25921
29. Ohno, H., Fournier, M. C., Poy, G., and Bonifacino, J. S. (1996) *J. Biol. Chem.* **271**, 29009–29015
30. Di Paolo, G., Pellegrini, L., Letinic, K., Cestra, G., Zoncu, R., Voronov, S., Chang, S., Guo, J., Wenk, M. R., and De Camilli, P. (2002) *Nature* **420**, 85–89
31. Cockcroft, S., and De Matteis, M. A. (2001) *J. Membr. Biol.* **180**, 187–194
32. Morgan, J. R., Di Paolo, G., Werner, H., Shchedrina, V. A., Pypaert, M., Pierbone, V. A., and De Camilli, P. (2004) *J. Cell Biol.* **167**, 43–50
33. Lee, S. Y., Voronov, S., Letinic, K., Nairn, A. C., Di Paolo, G., and De Camilli, P. (2005) *J. Cell Biol.* **168**, 789–799
34. Alonso, A., Sasin, J., Bottini, N., Nairn, A., Godzik, A., Hunter, T., Dixon, J., and Mustelin, T. (2004) *Cell* **117**, 699–711
35. Conner, S. D., Schroter, T., and Schmid, S. L. (2003) *Traffic* **4**, 885–890
36. Conner, S. D., and Schmid, S. L. (2002) *J. Cell Biol.* **156**, 921–929
37. Ricotta, D., Conner, S. D., Schmid, S. L., von Figura, K., and Honing, S. (2002) *J. Cell Biol.* **156**, 791–795
38. Thomas, S. M., and Brugge, J. S. (1997) *Annu. Rev. Cell Dev. Biol.* **13**, 513–609
39. Krauss, M., Kinuta, M., Wenk, M. R., De Camilli, P., Takei, K., and Haucke, V. (2003) *J. Cell Biol.* **162**, 113–124
40. Paleotti, O., Macia, E., Luton, F., Klein, S., Partisani, M., Chardin, P., Kirchhausen, T., and Franco, M. (2005) *J. Biol. Chem.* **280**, 21661–21666
41. Gong, L. W., Di Paolo, G., Diaz, E., Cestra, G., Diaz, M. E., Lindau, M., De Camilli, P., and Toomre, D. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 5204–5209
42. Sever, S. (2003) *Dev. Cell* **5**, 530–532