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Phosphatidylinositol phosphate 5-kinase Iβ recruits AP-2 to the plasma membrane and regulates rates of constitutive endocytosis

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Overexpression of phosphatidylinositol phosphate 5-kinase (PIP5KI) isoforms α, β, or γ in CV-1 cells increased phosphatidylinositol 4,5-bisphosphate (PIP2) levels by 35, 180, and 0%, respectively. Endocytosis of transferrin receptors, association of AP-2 proteins with membranes, and the number of clathrin-coated pits at the plasma membrane increased when PIP2 increased. When expression of PIP5KIβ was inhibited with small interference RNA in HeLa cells, expression of PIP5KIα was also reduced slightly, but PIP5KIγ expression was increased. PIP2 levels and internalization of transferrin receptors dropped 50% in these cells; thus, PIP5KIγ could not compensate for loss of PIP5KIβ. When expression of PIP5KIα was reduced, expression of both PIP5KIβ and PIP5KIγ increased and PIP2 levels did not change. A similar increase of PIP5KIα and PIP5KIβ occurred when PIP5KIγ was inhibited. These results indicate that constitutive endocytosis in CV-1 and HeLa cells requires (and may be regulated by) PIP2 produced primarily by PIP5KIβ.

Introduction

Lipid components of membranes play an important role in intracellular membrane traffic (Kobayashi et al., 1998; Ktistakis, 1998; Roth, 1999; Cockcroft and De Matteis, 2001; Cremona and De Camilli, 2001; Martin, 2001; Simonsen et al., 2001). Mutations that block secretion or endocytosis map to enzymes that modify lipids, particularly phosphatidylinositides, and many proteins that are important for vesicle formation and consumption contain modules that bind to specific lipids. In the process of endocytosis, many proteins important for assembling a clathrin-coated vesicle bind to phosphatidylinositol 4,5-bisphosphate (PIP2). These proteins include the AP-2 adaptor responsible for binding endocytic cargo (Gaidarov and Keen, 1999; Rohde et al., 2002), a brain-specific adaptor protein (AP180; Ford et al., 2001), epsin, which recruits clathrin and curves membranes (Ford et al., 2002), and dynamin, a GTPase that functions in the transformation of a clathrin-coated bud into a vesicle (Lin et al., 1997; Achiriloaie et al., 1999; Vallis et al., 1999). Reagents that sequester PIP2 inhibit clathrin-mediated endocytosis in vitro (Jost et al., 1998), and mutant epsin (Itoh et al., 2001) or AP-2 mu2 (Rohde et al., 2002) unable to bind PIP2 are dominant inhibitors of endocytosis. Clathrin coats also contain a phosphatidylinositol 5-phosphatase, synaptojanin (McPherson et al., 1996), and nerve terminals in mice lacking synaptojanin appear to have defects in the uncoating of clathrin-coated vesicles (Cremona et al., 1999). Similarly, mutants of *Caenorhabditis elegans* with defects in synaptojanin have defects in both the budding and uncoating of clathrin-coated vesicles (Harris et al., 2000). Thus, PIP2 appears to regulate the assembly and disassembly of a clathrin coat.

PIP2 might function for clathrin-mediated endocytosis in several ways. PIP2 might serve as a membrane identity marker, allowing proteins important for endocytosis to collect at the correct membrane surface. In this event, PIP2 might control where endocytosis occurs without influencing the rates of endocytosis, if the steady-state concentration of PIP2 at the plasma membrane supports maximum rates of endocytosis. PIP2, either directly or after phosphorylation to PIP3 (Gaidarov et al., 1996, 2001; Rapoport et al., 1997), might also act as an allosteric regulator of proteins important for endocytosis, and thus modulate the endocytic rate. Phosphatidylinositides including PIP2 increase the affinity of AP-2 proteins for the internalization signals in endocytic cargo (Rapoport et al., 1997), and the crystal structure of the

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Key words: phosphatidylinositol kinase; endocytosis; clathrin; transferrin receptor; phosphatidylinositol 4,5-bisphosphate

Abbreviations used in this paper: HA, hemagglutinin; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP5KI, phosphatidylinositol phosphate 5-kinase; siRNA, small interference RNA.

AP-2 tetramer suggests that binding to phosphoinositides may be required to open the binding site for internalization signals (Collins et al., 2002).

In addition to proteins of the clathrin coat, many other proteins bind PIP2 at the plasma membrane, and there must exist some mechanism regulating competition between different processes. One way to accomplish this would be to increase the production of PIP2 locally by controlling the location and activity of a phosphatidylinositol phosphate kinase. Interactions between the kinase and components of a particular cellular function that is regulated by PIP2 would then serve to channel PIP2 into the pathway subserving that function.

Two classes of lipid kinases produce PIP2 (Fruman et al., 1998). Originally classified as PI4P5K type I and type II, type I enzymes were found to phosphorylate the 5 position of the abundant phosphatidylinositol 4-phosphate to produce PIP2, but type II enzymes are really PIP 4-kinases that produce PIP2 by phosphorylating the 4 position of phosphatidylinositol 5-phosphate, a lipid species about which little is currently known. There are three isoforms of type I enzymes; phosphatidylinositol phosphate 5-kinase (PIP5KI) α, β, and γ. The mouse and human PIP5KIα and β enzymes were named in a reciprocal manner (Ishihara et al., 1996; Loijens and Anderson, 1996), and in this report we will use the human nomenclature throughout. PIP5KI enzymes contain a highly conserved central catalytic domain of ~400 residues and have nonconserved amino- and carboxyl-terminal sequences. The terminal domains contain information for dimerization of the enzyme (Galiano et al., 2002), and may serve other isoform-specific functions. PIP5KI enzymes and their yeast counterpart are negatively regulated by phosphorylation (Vancurova et al., 1999; Park et al., 2001). Two splice variants of PIP5KIγ are produced, and the longer form, which predominates in brain, is found at adhesion plaques as well as at the plasma membrane (Di Paolo et al., 2002; Ling et al., 2002). PIP5KIα and γ have been implicated in different specialized forms of endocytosis. PIP5KIγ is the major producer of PIP2 at the synapse, a site of extremely active endocytosis (Wenk et al., 2001). A truncated form of PIP5KIα was identified through a genetic screen for cDNAs that could restore signaling through a mutant CSF-1 receptor (Davis et al., 1997). The truncated kinase apparently acted as a dominant-negative inhibitor of endocytosis, allowing the mutant receptor to persist at the plasma membrane. Overexpressed PIP5KIα increased endocytosis of activated EGF receptors and was coprecipitated with the receptors, whereas PIP5KIβ was reported to have no effect on endocytosis of EGF (Barbieri et al., 2001). These observations are consistent with the ability of PIP5KI enzymes to regulate clathrin coat formation for at least certain endocytic events, and suggest that different isoforms may function in different cell types or for different endocytic activities.

Both endocytosis at the synapse and regulated endocytosis of growth factor receptors differ in a number of ways from the constitutive endocytosis of nutrient receptors, such as the transferrin receptor. Both use additional components compared with constitutive endocytosis. Endocytosis of synaptic vesicle components is an order of magnitude faster than clathrin-mediated endocytosis in other cell types, and the rate of endocytosis of growth factor and hormone receptors is acutely regulated. Thus, we were interested in determining if the rates of constitutive endocytosis might be regulated by PIP2 levels, and if so, by which enzyme. Using overexpression and RNAi to raise or lower the cellular concentration of each of the PIP5KI isoforms in HeLa or CV-1 cells, we found that increased PIP2 levels increased the rate of constitutive endocytosis. PIP5KIβ was the major contributor to PIP2 levels and to clathrin-mediated endocytosis of the transferrin receptor. In contrast, increased expression of exogenous PIP5KIγ or increased transcription of endogenous PIP5KIγ had no impact on endocytosis or cellular PIP2 levels.

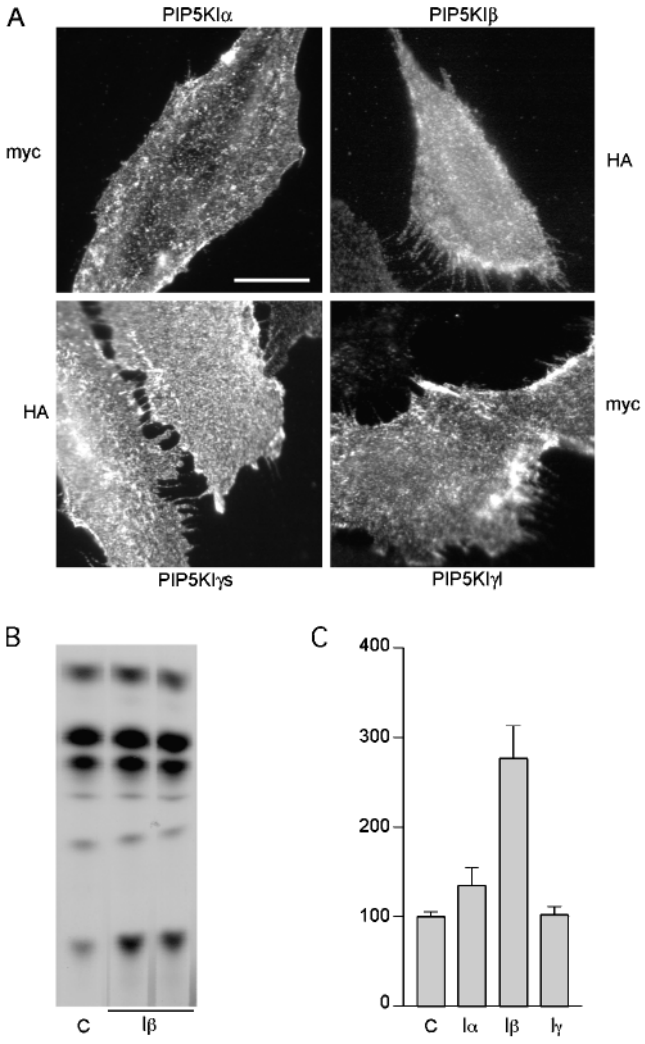

Figure 1. **PIP5KI is effectively overexpressed by recombinant adenoviruses or transfection.** (A) CV1 cells infected with adenovirus expressing PIP5KIα or β or transfected with plasmids expressing either the long (l) or short (s) forms of PIP5KIγ were labeled with antibodies for HA or myc tags as indicated. The overexpressed isoforms all localized at the plasma membrane. (B) Cells treated as in A were ^{32}P-labeled (4 h) and their lipids were extracted. Equal counts of lipids were resolved by TLC, and the bottom spot corresponding to PIP2 was quantified by densitometry. An example of the image of a TLC plate showing samples from uninfected cells (C) and two samples expressing PIP5KIβ (Iβ) is shown. (C) Averages of PIP2 levels in three or more samples of cells expressing each of the three PIP5KI isoforms are graphed relative to the control (C = 100) with SD.

Results

The effects of increased expression of PIP5KI enzymes on internalization of transferrin receptors

To increase the expression of PIP5KI isoforms, CV-1 cells were infected with replication-defective adenoviruses expressing PIP5KIα or β, or were transiently transfected with plasmid vectors to express the 87- or 90-kD variants of the γ isoform. Cells were stained with antibodies to the epitope tags on the recombinant proteins to determine the percentage of cells expressing them and the locations of the proteins within the cells. PIP5KI isoforms were effectively overexpressed after either adenoviral infection (>85% efficiency) or transient transfection (~20% efficiency). All three overexpressed isoforms located primarily at the plasma membrane (Fig. 1 A). To circumvent the low efficiency of transfection of the plasmid expressing PIP5KIγ and to be able to perform biochemical experiments, we cotransfected the PIP5KIγ expression plasmid with a GFP expression plasmid at a 3 to 1 ratio, and determined that 97% of cells expressing GFP also expressed PIP5KIγ by fluorescence microscopy. Then, we sorted the GFP-positive cells by flow cytometry one day after transfection and performed biochemical experiments on the sorted cells one day after they were returned to culture.

After infection or transfection, cells were labeled with [^{32}P]orthophosphate, and lipids were extracted and analyzed by TLC (Fig. 1 B). The three isoforms of PIP5KI increased PIP2 production to different extents. Overexpression of PIP5KIα and β resulted in about a 35 and 180% increase in PIP2 (Fig. 1 C), respectively, and γ had no effect. However, the PIP5KIγ expression vector that we used did cause the formation of actin comets when transfected into REF52 cells (unpublished data). Actin comets are produced in REF52 cells transfected with PIP5KIα or β (Rozelle et al., 2000); thus, the PIP5KIγ produced by our vectors was active in other cellular backgrounds.

Internalization assays were performed to determine whether PIP5KI expression levels would affect constitutive endocytosis. The expression of transferrin receptors in cells expressing PIP5KI proteins was examined by immunoblotting (unpublished data) and by fluorescence microscopy (Fig. 2 A), and was not changed compared with control cells, although the cells overexpressing PIP5KIβ became more elongated than uninfected cells. Overexpression of either the 87-kD (unpublished data) or 90-kD γ isoforms had no effect on endocytosis of transferrin receptors, but overexpression of PIP5KIα and β increased the rate of endocytosis of transferrin ~30 and 100%, respectively (Fig. 2). To determine whether the observed differences between the α and β isoforms were due to differences in protein expression levels, we immunoprecipitated the overexpressed proteins from lysates of cells ^{35}S-labeled with amino acids. We found that both proteins were expressed at a very similar level (unpublished data).

Expression of PIP5KIβ increases internalization of influenza virus hemagglutinin (HA) Y543

To determine if increased expression of PIP5KIβ stimulated constitutive endocytosis in general, we measured internalization of a second protein, the HA Y543 mutant. This HA contains a cysteine-to-tyrosine mutation in a very short (10 amino acid) cytoplasmic domain that allows HA Y543 to be internalized by clathrin-coated pits (Lazarovits and Roth, 1988). When compared with cells infected with a GFP adenovirus, cells infected with a PIP5KIβ adenovirus had increased internalization of HA Y543 similar to the increased rate observed for the transferrin receptor (Fig. 3). Thus, the effect of increased expression of PIP5KIβ on endocytosis is not specific for a particular receptor.

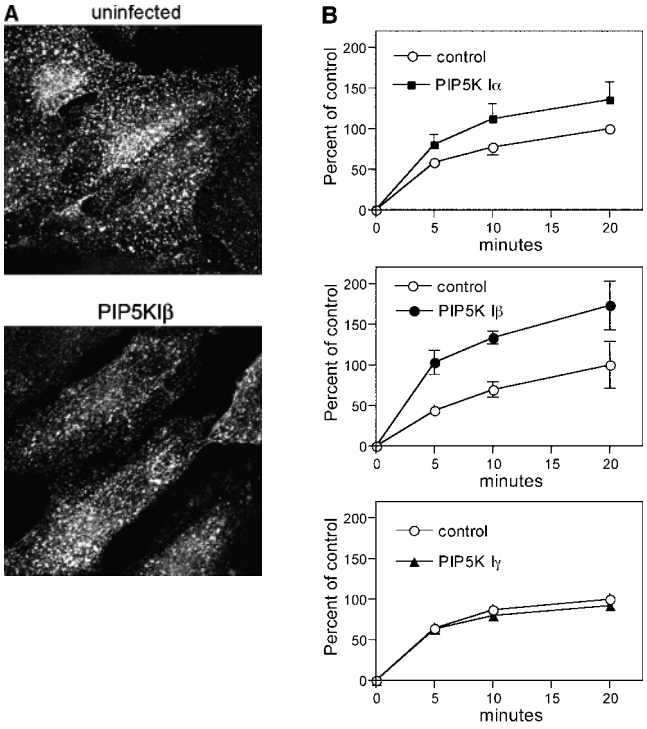

Figure 2. **Transferrin internalization is increased in cells overexpressing PIP5KIα or β, but not γ.** (A) Uninfected or CV-1 cells infected with adenovirus expressing PIP5KIβ were allowed to bind fluorescent transferrin. Images of the cells were taken with a confocal microscope (model 510; Carl Zeiss MicroImaging, Inc.) at identical settings. Infected and uninfected cells bound similar amounts of transferrin. (B) Transferrin endocytosis was measured in cells with overexpressed PIP5KI isoforms as described in Materials and methods. Averages of three independent experiments with SD are shown, except in the bottom panel, in which a representative experiment of two is shown.

Drugs that disrupt the actin cytoskeleton do not antagonize transferrin uptake in cells overexpressing PIP5KI

Because PIP5KI has been reported to stimulate formation of actin stress fibers and affect cell shape (Yamamoto et al., 2001), we investigated the possibility that the increase in endocytosis could be a consequence of changes in the organization of the actin cytoskeleton. We tested the effect of two toxins, cytochalasin D and latrunculin A, on transferrin uptake in both control cells and in cells overexpressing PIP5KIβ. Both of these toxins result in actin filament disassembly; cytochalasin D by capping actin filaments and latrunculin A by sequestering actin monomers (Coue et al.,

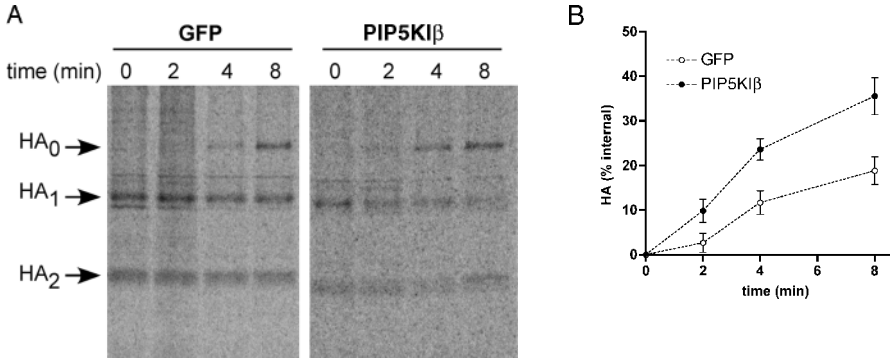

Figure 3. **Overexpression of PIP5KIβ increases the rate of internalization of HA Y543.** CV1 cells were infected with adenovirus encoding PIP5KIβ or GFP (control). After 24 h, both samples were infected with recombinant SV40 virus expressing HA Y543. 26 h after the SV40 infections, the cells were ^{35}S-labeled with amino acids, and internalization of HA was measured for the indicated times as described in Materials and methods. A shows the results of a representative experiment and B shows the quantification of four experiments. Error bars in B are standard errors.

1987; Sampath and Pollard, 1991). Overexpression of PIP5KIβ resulted in increased endocytosis to the same extent either in the presence or absence of cytochalasin D (Fig. 4) or latrunculin A (unpublished data). Thus, the increase in endocytosis caused by overexpression of PIP5KIβ was not related to the previously described increase in stress fibers (Yamamoto et al., 2001).

Overexpression of PIP5KI increases the number of clathrin-coated pits and the proportion of membrane-associated AP-2

The ways in which transferrin uptake could be increased would be to increase the density of transferrin receptors packed into each coated pit, to increase the numbers of coated pits that form, or to increase the speed with which coated pits form and bud off. Thus, we examined how overexpression of PIP5KI would affect the number of clathrin-coated pits at the plasma membrane and the proportion of AP-2 adaptor proteins that were bound to membranes. Analysis by EM revealed that overexpression of PIP5KIα or β increased the number of clathrin-coated pits at the plasma membrane by 0.7- and 1.7-fold, respectively (Table I). Immunoblots of cytosolic and membrane fractions from cells overexpressing either isoform showed that the fraction of α-adaptin bound to membranes had increased (Fig. 5). Together, these results show that the level of PIP2 in CV-1 cells produced by endogenous PIP5KI enzymes under the growth conditions of our experiments is rate limiting for endocytosis, and thus the rates of constitutive endocytosis might be regulated through production of PIP2.

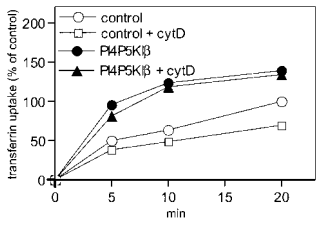

Figure 4. **Cytochalasin D does not abolish the increased endocytosis observed in cells overexpressing PIP5KIβ.** CV1 cells were infected with adenovirus expressing PIP5KIβ as in Fig. 1. Before measuring endocytosis, cells were treated with 5 μM cytochalasin D for 30 min. Cells were also stained with phalloidin to confirm that the actin cytoskeleton was disrupted (not depicted).

Expression of PIP5KI proteins is coordinately regulated

To determine which of the PIP5KI isoforms is required for constitutive endocytosis, expression of each was inhibited by small interference RNA (siRNA) oligonucleotides in HeLa cells. We observed that on the second day after transfection with siRNA oligonucleotides specific for PIP5KIβ, cells appeared to be healthy, but their growth rate had slowed. Therefore, the effects of siRNA on the steady-state levels of protein of each of the three PIP5KI isoforms were measured after two days by immunoblotting. A representative immunoblot from an experiment targeting PIP5KIα expression is shown in Fig. 6, with quantification of the averages of three such experiments. Expression was reduced by 75, 50, and 90% for the α, β, and γ isoforms, respectively. Only the siRNA specific for PIP5KIβ decreased PIP2 production, and did so by ~50% (Fig. 6 B). However, when mRNA for each of the three PIP5KI isoforms was measured in cells treated with siRNA for one of them, we observed that there were changes in the expression of the other two PIP5KI enzymes (Fig. 6 C). RNAi targeting PIP5KIβ also slightly reduced PIP5KIα and induced a threefold increase in expression of PIP5KIγ. Similar to the overexpression experiments, this stimulation of mRNA for endogenous PIP5KIγ did not result in an increase in total PIP2 production (Fig. 6 B). The small decrease in PIP5KIα was caused by three different siRNA oligonucleotides specific for regions of PIP5KIβ that differed from PIP5KIα (unpublished data). In contrast, siRNA specific for PIP5KIα caused almost a twofold increase in mRNA for both PIP5KIβ and γ. siRNA specific for PIP5KIγ caused a similar increase in mRNA for the other two isoforms. Measured by immunoblotting, changes in protein levels of the other two isoforms in cells in which one was targeted by

Table I. **Overexpression of PIP5KI increases the number of clathrin-coated pits**

	Control	PIP5KIα	PIP5KIβ
Exp 1	0.055 (34)	0.091 (24)	0.153 (52)
Exp 2	0.061 (23)	0.102 (35)	0.161 (43)

Cells infected with adenovirus-encoding β-galactosidase (Control) and PIP5KIα and PIP5KIβ were processed for EM. Clathrin-coated pits were counted and expressed as coated pit/micrometer of plasma membrane (see Materials and methods). Numbers in parenthesis represent number of coated pits counted for each sample. Data from two experiments are shown.

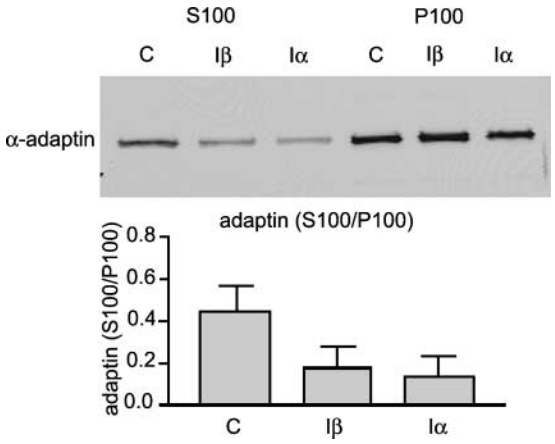

Figure 5. **Cells overexpressing PIP5KIα or β have more AP-2 on membranes.** Cytosol (S100) and crude membrane fractions (P100; see Materials and methods) from cells overexpressing PIP5KI enzymes or β-galactosidase (C) were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and blotted with antibody specific for α-adaptin. An example of such a blot is shown. Averages from three independent experiments are graphed with SD.

siRNA were less than the changes in mRNA, but the patterns were similar (unpublished data). As is discussed in more detail in the Discussion, these results indicate that gene expression of each PIP5KI isoform is sensitive to the expression of the other isoforms. However, the γ isoform does not compensate for loss of α or β, even when significantly overexpressed.

Because of this unexpected result, we investigated the effect of overexpressing a single PIP5KI isoform on the expression of the other two isoforms by Western blot analysis (Fig. 7). Overexpressing PIP5KIβ decreased PIP5KIα and γ by 40%. Increased PIP5KIγ reduced PIP5KIα ~30%, but had no effect on PIP5KIβ. In contrast, overexpressing PIP5KIα did not significantly change the expression levels of PIP5KIβ or γ. Thus, both overexpression and knockdown experiments indicate that the expression of the three PIP5KI enzymes is interdependent, but the regulation is complex and the three isoforms are obviously not equivalent.

A reduction in cellular PIP2 production decreases endocytosis of transferrin receptors

In cells treated with siRNA oligonucleotides that targeted PIP5KIβ, transferrin internalization was reduced by 40%, whereas siRNA specific for PIP5KIα or γ did not have a significant effect (Fig. 8 A). This result was consistent with the reduction of PIP2 production in cells in which levels of PIP5KIβ were lowered, but no change was observed in PIP2 in cells in which the other isoforms were lowered. This effect was not due to a general toxic effect of loss of PIP5KIβ, because under identical conditions, the rate of movement of the influenza virus HA through the early secretory pathway was not decreased (unpublished data). In the case of increased PIP2 production in cells overexpressing PIP5KIβ, we observed a decrease in the ratio of cytosolic-to-membrane-bound AP-2 adaptors (Fig. 5). In contrast, in cells treated with siRNA in which expression of PIP5KIβ as well as PIP2 levels decreased, the ratio of cytosolic to membrane-bound AP-2 increased twofold (Fig. 8 B). Thus, it is possible that the decrease in the rate of endocytosis in cells in which PIP2 is lowered is due, at least in part, to decreased association of AP-2 with membranes.

Discussion

Three isoforms of type I PIP5K have been described; PIP5KIα, β, and γ (Ishihara et al., 1996, 1998; Loijens and Anderson, 1996). PIP5KIα, but not β, has been reported to be required for endocytosis of the EGF receptor (Barbieri et al., 2001) and to have effects on endosomes (Galiano et al., 2002). PIP5KIγ is the major producer of

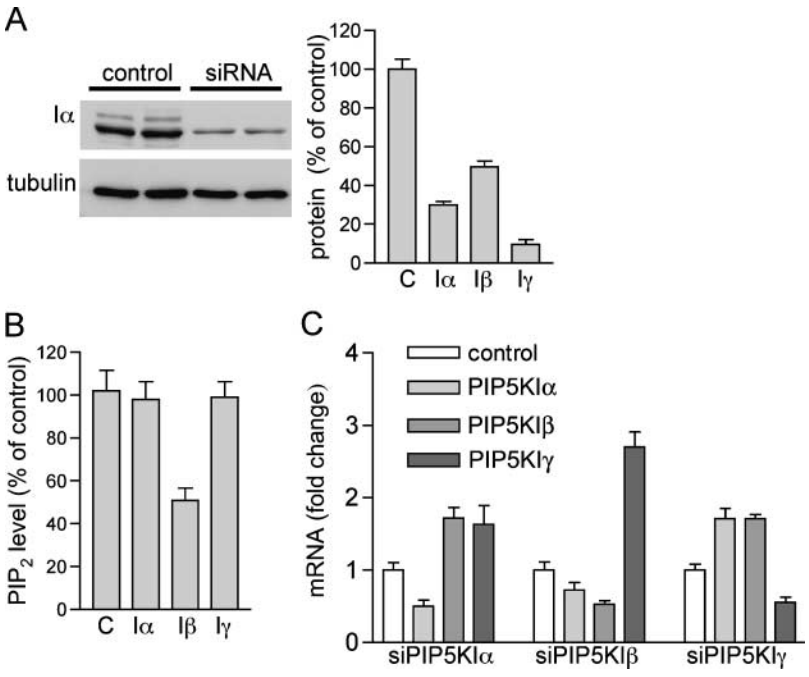

Figure 6. **Small interfering RNA specific for PIP5KIβ (but not α or γ) reduces PIP2 levels in HeLa cells.** HeLa cells were transfected with siRNA oligos targeting the α, β, or γ isoforms of PIP5KI or an irrelevant sequence (control, C). Western blots with antibodies specific for the respective protein and tubulin (loading control) were performed 48 h after transfection. (A) An example of a Western blot specific for PIP5KIα is shown. Similar blots were quantified by densitometry, and the values are presented graphically. (B) PIP2 levels in cultures transfected with siRNAs specific for the enzymes shown were measured as described in Fig. 1 B. (C) mRNA for each enzyme in cells treated with siRNA specific for one isoform were measured by real-time PCR. For each isoform, values in A and B are expressed as a percentage of the value measured in the control transfected with unrelated siRNA. All graphs present the averages of at least three experiments with SD.

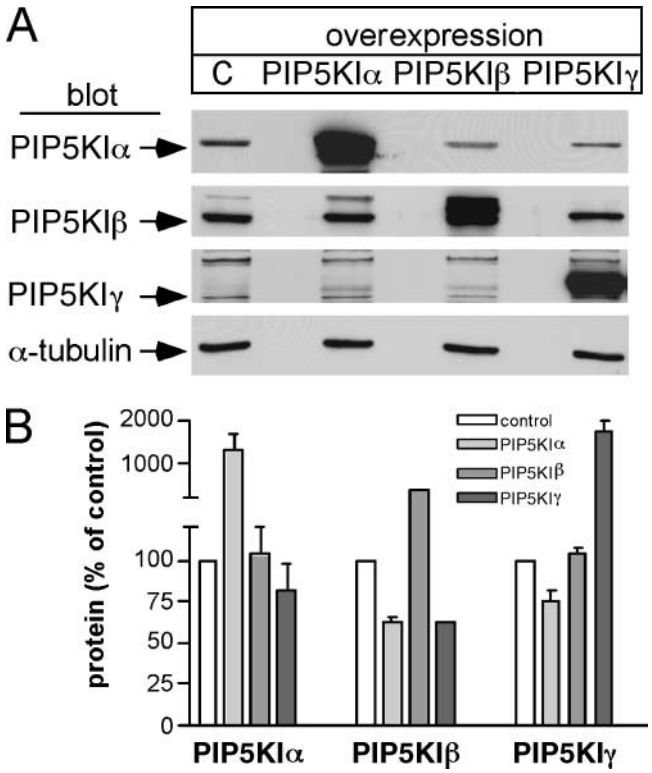

Figure 7. **Overexpression of a PIP5KI enzyme affects the expression of the other isoforms.** (A) Cell lysates obtained from CV1 cells individually overexpressing each of the three isoforms of PIP5KI or GFP (control, C) were subjected to SDS-PAGE and immunoblotted with antibodies to the three isoforms of PIP5KI and to tubulin as an internal control. (B) Blots were quantified by densitometry, and the results are expressed as percentage of protein in experimental samples compared with the amount of each protein in the control sample.

PIP2 in the synapse (Wenk et al., 2001), where PIP2 is required for the recycling of synaptic vesicle membranes (Cremona et al., 1999). In contrast, we find that PIP5KIβ plays a major role in constitutive receptor-mediated endocytosis in CV-1 cells and HeLa cells. Our results show that the rates of constitutive endocytosis can be both increased and decreased by changes in PIP2 levels, which suggests that constitutive endocytosis may be regulated through changes in the activity or location of PIP5KI enzymes, and perhaps other proteins that activate PIP5KI or that supply its substrate, PI4P.

We observed that overexpression of recombinant PIP5KIγ (both splice variants) did not change PIP2 levels or impact constitutive endocytosis, and the increase in endogenous PIP5KIγ that occurred when expression of PIP5KIβ was inhibited by siRNA did not compensate for the loss of PIP5KIβ. Therefore, it is likely that PIP5KIγ plays no role in constitutive endocytosis in either CV-1 cells or HeLa cells. Either this isoform is not active in these cell types, or it acts in a location different from the plasma membrane that does not provide a significant fraction of the total PIP2 production in these cells. We can conclude less about PIP5KIα. Overexpressing this isoform had less impact on PIP2 levels than did overexpressing PIP5KIβ, although protein levels of PIP5KIα were slightly higher. A possibility consistent with our observations and those reported by Barbieri et al. (2001) is that a significant fraction of PIP5KIα may be sequestered and not available for constitutive endocytosis, perhaps by associating with growth factor receptors. Perhaps this fraction of PIP5KIα is not constitutively active, and only the remaining PIP5KIα is able to produce PIP2 and impact constitutive endocytosis. We cannot answer the question of whether PIP5KIα plays some role in constitutive endocytosis or can compensate for the loss of PIP5KIβ, because siRNA oligonucleotides that inhibited PIP5KIβ also lowered expression of PIP5KIα. Three separate oligonucleotide sequences of PIP5KIβ that contained significant mismatches with PIP5KIα each had this effect, and we currently have no explanation for this.

Also, we observed that inhibiting the expression of one isoform of PIP5KI with siRNA resulted in increased expression of the other isoforms and, conversely, overexpression of one isoform can result in reduced expression of the other isoforms. Thus, transcription of PIP5KI genes is coordinately regulated in some fashion. In the case of siRNA ablation of PIP5KIβ, which also slightly lowered PIP5KIα, PIP5KIγ mRNA increased threefold, but this did not translate into an active enzyme capable of rescuing total cellular PIP2 levels (Fig. 6). PIP2 levels did not decrease when PIP5KIα expression was lowered by siRNA, presumably because the increased expression of PIP5KIβ compensated for the loss of α. Inhibition of expression of PIP5KIγ also

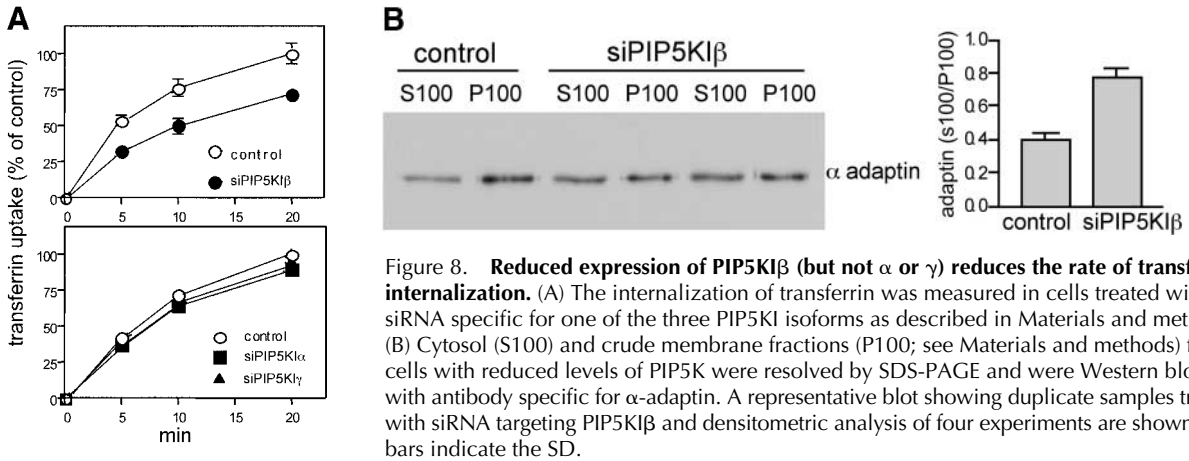

Figure 8. **Reduced expression of PIP5KIβ (but not α or γ) reduces the rate of transferrin internalization.** (A) The internalization of transferrin was measured in cells treated with siRNA specific for one of the three PIP5KI isoforms as described in Materials and methods. (B) Cytosol (S100) and crude membrane fractions (P100; see Materials and methods) from cells with reduced levels of PIP5K were resolved by SDS-PAGE and were Western blotted with antibody specific for α-adaptin. A representative blot showing duplicate samples treated with siRNA targeting PIP5KIβ and densitometric analysis of four experiments are shown. Error bars indicate the SD.

caused a twofold increase in the other two isoforms. Because we have no evidence that PIP5KIγ is active in HeLa cells, either there is a mechanism that senses the presence of PIP5KI isoforms independently of their enzymatic activity, which suggests that they might share common interaction partners, or the sensing mechanism uses a PIP2 pool that is a small fraction of total cellular PIP2.

One difficulty for understanding the functions for PIP2 in endocytosis is that it interacts with a great variety of factors, including lipid-modifying enzymes, such as PLD1 (Liscovitch et al., 1994), and the actin cytoskeleton (Yin and Janmey, 2003), which plays an undefined role in constitutive receptor-mediated endocytosis (Fujimoto et al., 2000). Overexpression of any of the three isoforms of PIP5KI leads to reorganization of the actin cytoskeleton (Shibasaki et al., 1997; Ishihara et al., 1998; Rozelle et al., 2000; Yamamoto et al., 2001). In particular, overexpression of the PIP5KIβ isoform in CV-1 cells promotes formation of stress fibers, and cells become elongated (Yamamoto et al., 2001). We have shown that actin-disrupting drugs did not prevent the PIP5KI-mediated increase in endocytosis of transferrin receptors; thus, the effects of PIP5KI for promoting stress fibers is separate from its effect on endocytosis. Our results suggest that one effect of PIP5KIβ on constitutive endocytosis is to increase the fraction of endocytic adaptors bound to membranes that may in turn regulate the formation of clathrin-coated pits. We observed that the proportions of AP-2 proteins associated with membranes increased with increasing PIP2 levels. Cells overexpressing PIP5KIβ also had more clathrin-coated pits at the plasma membrane. This observation suggests that the impact of increased PIP2 for increasing endocytic rates was preferentially on the processes for forming clathrin-coated pits, rather than those responsible for budding off clathrin-coated vesicles or that determine the occupancy of transferrin receptors in coated pits.

Materials and methods

Reagents and antibodies

Alexa®-labeled transferrin was purchased from Molecular Probes, Inc. Lipid standards were purchased from Avanti Polar Lipids, Inc. [^{32}P]orthophosphoric acid was purchased from NEN Life Science Products. All other chemicals were purchased from Sigma-Aldrich unless otherwise specified. Monoclonal anti α-adaptin was purchased from BD Biosciences, monoclonal anti-HA epitope tag was purchased from BAbCO, anti-myc was purchased from Santa Cruz Biotechnology, Inc. Antibody to PIP5KIα was purchased from Santa Cruz Biotechnology, Inc., polyclonal anti-PIP5KIβ was provided by C. Carpenter (Harvard University, Cambridge, MA), anti-PIP5KIγ was provided by Dr. P. De Camilli (Yale University, New Haven, CT), and polyclonal anti-HA was produced in the Roth laboratory.

Transfection, adenovirus, and SV40 infections

Recombinant adenovirus expressing an HA-tagged version of mPIP5KIα was obtained from Y. Shibasaki (University of Tokyo, Tokyo, Japan). Adenoviruses expressing the myc-tagged type PIP5KIα, β-galactosidase, or GFP were constructed using the AdEasy™ Adenoviral Vector System (Stratagene). A plasmid encoding the 90-kD splice variant of PIP5KIγ was obtained from the Kazusa DNA Research institute (KIAA0589), myc-tagged and subcloned into pCMV5. A cDNA encoding an HA-tagged 87-kD splice variant of mPIP5KIγ (Ishihara et al., 1998) was obtained from Dr. P. De Camilli with permission of Dr. Y. Oka (Tohoku University, Sendai, Japan), and was expressed using the plasmid vector pcDNA3.1. For all experiments using adenovirus vectors, cells were infected at a multiplicity of infection of 10. After 2 h, cells were washed and then cultured in fresh DME containing 10% FCS for 36 h. For experiments expressing the HA Y543 mutant, cells were infected with recombinant SV40 virus in suspension for 30 min on ice followed by culture in complete growth medium for 26 h. For experiments using transient transfection, cells were transfected with pCMV5-PIP5KIγ and pEGFP-C3 (CLONTECH Laboratories, Inc.) at a ratio of 3 to 1, using LipofectAMINE™ according to the manufacturer's instructions (Invitrogen).

RNA interference

siRNA oligonucleotides were designed according to the protocol provided by Dharmacon Research, Inc. In brief, sequences of the type AA(N19) (N = any nucleotide) from the ORF of the targeted mRNA were selected and subjected to a BLAST® search (National Center for Biotechnology Information database) against the human genome sequence to ensure the specificity of targeting. RNA oligonucleotides encoding both the sense and antisense of the target were synthesized by the Center for Biomedical Inventions (University of Texas Southwestern Medical Center at Dallas, Dallas, TX), and annealed after a protocol from Dharmacon Research, Inc. The siRNA sequence targeting PIP5KIα (GenBank/EMBL/DDBJ accession no. U78575) was from position 1923–1943. The siRNA targeting PIP5KIβ (GenBank/EMBL/DDBJ accession no. NM_003558) encoded bases 1114–1135. The oligonucleotide targeting PIP5KIγ (GenBank/EMBL/DDBJ accession no. XM_047620) encoded bases 619–639. An oligonucleotide corresponding to nucleotides 695–715 of the firefly luciferase (GenBank EMBL/DDBJ accession no. U31240) was used as a negative control. On d 1, HeLa cells were plated in 6-well plates at 30–40% confluency in antibiotic-free DME supplemented with 10% (vol/vol) FCS, 10 mM Hepes, and 1 mM sodium pyruvate. On d 2, siRNA was introduced into cells using OligofectAMINE™ reagent according to the manufacturer's instructions (Life Technologies), with 10 μl of 20 μM siRNA and 4 μl transfection reagent/well. On d 4, cells were lysed and analyzed by Western blotting.

Quantitative real-time PCR

Total RNA was extracted from HeLa cells transfected with siRNAs using the total RNA/mRNA isolation reagent RNA STAT-60™ (TEL-TEST, Inc.). RNA samples were treated with DNase I (RNase-free; Roche), and were reverse-transcribed with random hexamers using SuperScript™ II RNase H-reverse transcriptase to generate cDNA. Primer Express software (PerkinElmer) was used to design primers for cyclophilin (used as internal control; GenBank/EMBL/DDBJ accession no. XM_057194), forward TGCCATCGCCAAGGAGTAG, reverse TGC ACA GAC GGT CAC TCA AA; PIP5KIα, forward TCAAAGGCTCAACCTACAAACG, reverse TTTAAATGTGGGAAGAGGCTTCT; PIP5KIβ, forward GAAACGGTGCAATTCAATCG, reverse TCCTGGCTAATTGAGGACACA; and PIP5KIγ, forward TGTCGCCTTCCGCTACTTC, reverse GGCTCATTGCACAGGGAGTAC. Primers were validated through analysis of template titration and dissociation curves to establish both linearity of the reaction and production of a single product. PCR assays were conducted on a sequence detection system (Prism® 7000; Applied Biosystems). The 20-μl final reaction volume contained 50 ng reverse-transcribed RNA, 150 nM of each primer, and 10 μl SYBR® Green PCR Master Mix (Applied Biosystems). PCR reactions were performed in triplicate, and relative RNA levels were determined by the comparative Ct method (User Bulletin No. 2; PerkinElmer).

Membrane fractionation

Cells were homogenized in 0.2 M sucrose buffered with 20 mM Hepes (pH 7.3) by 25 strokes in a pre-chilled steel homogenizer, and were centrifuged at 960 *g* for 15 min at 4°C. The supernatant was then centrifuged at 128,000 *g* for 60 min at 4°C. Supernatants and pellets were separated and stored at −80°C.

Endocytosis assays

Cells infected with adenoviruses or transfected with RNAi oligos were rinsed and incubated in serum-free medium for 30 min to remove any residual transferrin, and then were exposed to 50 μg/ml transferrin conjugated with Alexa® Fluor 488 or 633 at 37°C for the times indicated. Internalization was stopped by chilling the cells on ice. External transferrin was removed by washing with ice-cold serum-free DME and PBS, whereas bound transferrin was removed by an acid wash in PBS at pH 5.0 followed by a wash with PBS at pH 7.0. The fluorescence intensity of internalized transferrin was measured by flow-cytometry using FACScan™ or FACSCalibur™ (Becton Dickinson) instruments, and the average intensity of 10,000 cells was recorded for each time point. Data are normalized to the increase in mean fluorescence of cells infected with adenovirus expressing β-galactosidase. We confirmed that the uptake of fluorescent transferrin was receptor mediated by measuring the uptake of fluorescent transferrin in the presence of a 100-fold excess of nonfluorescent holotransferrin under the same experimental conditions. No increase in cell-associated fluorescence was

obtained in the presence of excess competing nonfluorescent transferrin. Cell viability was measured by staining with propidium iodide.

Internalization of influenza mutant HA Y543

Endocytosis of an internalization-competent HA mutant was measured as described previously (Lazarovits and Roth, 1988). In brief, CV1 cells were infected with recombinant SV40 virus encoding the HA Y543 mutant. 26 h after SV40 infection, cells were washed and ^{35}S-labeled with amino acids for 30 min at 37°C. The labeling medium was then replaced with DME, and after an additional incubation of 2 h at 37°C, the cells were exposed to polyclonal anti-HA antibody on ice for 45 min. Antibody was removed, cells were washed, and were then incubated at 37°C for 0, 2, 4, and 8 min. Cells were then returned to ice, treated with 100 μg/ml trypsin for 45 min, and lysed in the presence of 200 μg/ml soybean trypsin inhibitor and a cocktail of protease inhibitors. The HA protein bound to antibody was immunoprecipitated with protein A–Sepharose and was resolved by SDS-PAGE. The internalized HA was calculated as the fraction that became resistant to trypsin cleavage and was expressed as percentage of total HA.

PIP2 analysis

Cells were labeled with 40 μCi/ml [^{32}P]orthophosphoric acid for 4 h in phosphate-free DME plus 0.5% FBS. Lipids were extracted from the cells with a 4:10:5 mixture of $CHCl_3$:CH3OH:1N HCl and were resolved by TLC, visualized by autoradiography, and quantified by densitometry. Lipid standards (Avanti Polar Lipids, Inc.) were detected by iodine vapors.

Fluorescence microscopy

Cells on coverslips were washed with PBS and fixed in 3.7% PFA for 15 min. The fixative was removed and cells were washed with DME and permeabilized with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% gelatin, 5 mM EDTA, 0.02% NaN3, 0.05% NP-40, and 0.05% Triton X-100. Nonspecific binding sites were blocked with PBS containing 1% BSA before incubating samples with primary and secondary antibodies for 1 h at RT. Coverslips were mounted on glass slides with Aqua-Polymount (Polysciences) and visualized with a microscope (Axioplan; Carl Zeiss MicroImaging, Inc.) fitted with a confocal scanning head (MRC600; Bio-Rad Laboratories) and a Kr/Ar laser (Bio-Rad Laboratories) or with a confocal microscope (model 510; Carl Zeiss MicroImaging, Inc.).

EM

Control and infected cells were processed for EM following standard procedures using osmium tetroxide and lead citrate for staining. Clathrin-coated pits were visualized and counted at a magnification of 70,000 in an electron microscope (model 1200EX; JEOL USA, Inc.). Digital images of each cell were stored for analysis, and the length of plasma membrane on which coated pits were counted was quantified using Image J 1.27z software (National Institutes of Health, Bethesda, MD).

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