Dissecting the role of PtdIns(4,5)P₂ in endocytosis and recycling of the transferrin receptor

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Summary
Endocytosis and recycling of membrane proteins are key processes for nutrient uptake, receptor signaling and synaptic transmission. Different steps in these fission and fusion cycles have been proposed to be regulated by physiological changes in plasma membrane (PM) phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P₂] concentration. Here, we use a chemical enzyme-translocation strategy to rapidly reduce PM PtdIns(4,5)P₂ levels while monitoring clathrin-mediated endocytosis and recycling. PtdIns(4,5)P₂ hydrolysis blocked transferrin receptor endocytosis and led to a marked increase in the concentration of transferrin receptors in the PM, suggesting that endocytosis is more sensitive to changes in PtdIns(4,5)P₂ than recycling. Reduction of PM PtdIns(4,5)P₂ levels led to a near complete dissociation of Adaptor protein 2 (AP-2) from the PM but had only a small effect on clathrin assembly. This argues that receptor-mediated PtdIns(4,5)P₂ reduction preferentially suppresses AP-2-mediated targeting of cargo to endocytic sites rather than the assembly of clathrin coats or recycling of endocytic vesicles.

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Key words: Endocytosis, Phosphoinositides, Recycling

Introduction
Although phosphoinositides represent a minor fraction of cellular phospholipids, they are integral components of membranes and are believed to play key roles in cell signaling, cytoskeletal organization, vesicular transport and ion-channel regulation (Di Paolo and De Camilli, 2006; Suh and Hille, 2005). Among different phosphoinositide species, phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P₂] has been implicated in functioning as a second messenger that regulates clathrin-mediated endocytosis of receptors as well as recycling of internalized receptors back to the plasma membrane (PM) (Roth, 2004; Haucke, 2005; Di Paolo and De Camilli, 2006; Kim et al., 2006; Hinrichsen et al., 2003; Motley et al., 2003).

Although such a role of PtdIns(4,5)P₂ in endocytosis and recycling has been supported by a number of genetic and biochemical studies, PtdIns(4,5)P₂ has equally prominent roles as a cytoskeletal regulator and as a precursor for other second messengers, making it difficult to demonstrate that the observed effects of PtdIns(4,5)P₂ perturbations are direct. Long-term perturbations of PtdIns(4,5)P₂ levels might also cause changes in phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P₃], diacylglycerol and Ca²⁺ second messengers that alter gene expression and thereby indirectly interfere with endocytosis and recycling.

To overcome this limitation, we employed a rapamycin-induced hetero-oligomerization strategy to rapidly reduce the PM PtdIns(4,5)P₂ concentration within seconds. We and others have recently developed this method to test for a direct role of PtdIns(4,5)P₂ in ion-channel regulation (Suh et al., 2006), recruitment of proteins with polybasic residues (Heo et al., 2006), calcium signaling (Varmai et al., 2006) and endocytosis (Varmai et al., 2006; Zoncu et al., 2007). In this approach, PtdIns(4,5)P₂ is rapidly dephosphorylated by chemically inducible targeting of Inp54p, a PtdIns(4,5)P₂-specific 5’ phosphatase (Tsujishita et al., 2001), to the PM. The addition of iRap, a rapamycin analog (Inoue et al., 2005), causes dimerization of a PM-localized FKBP12-rapamycin binding domain (FRB) and a cytosolic Inp54p phosphatase domain conjugated to CFP and FKBP (CF-Inp) (Suh et al., 2006).

Here, we use this strategy to dissect the role of PtdIns(4,5)P₂ both in the internalization and recycling of transferrin receptors. We found that, although rapid reduction in PtdIns(4,5)P₂ concentration induced a near complete block of transferrin receptor endocytosis, bulk-phase endocytosis was not inhibited. Furthermore, the reduction in PtdIns(4,5)P₂ also enhanced the concentration of transferrin receptor at the PM, suggesting a preferential role of PtdIns(4,5)P₂ in regulating endocytosis over recycling. We further show that the clustering of transferrin receptors into coated pits is disrupted upon reduction of PtdIns(4,5)P₂ levels. PtdIns(4,5)P₂ reduction also dissociated the adaptor proteins AP-2, epsin and clathrin assembly lymphoid myeloid protein (CALM) from the PM, which is consistent with a role of PtdIns(4,5)P₂ in regulating AP-2 and possibly other adaptors in clustering transferrin receptors. Surprisingly, we found that this reduction in PtdIns(4,5)P₂ concentration has only a weak effect on the dynamic assembly of clathrin at the PM, suggesting that PtdIns(4,5)P₂ regulation is less important for the formation of clathrin coats. Together, our study suggests that reduction in PM PtdIns(4,5)P₂ concentration, as expected to occur upon receptor stimulation, first suppresses the adaptor-mediated targeting of cargo to endocytic sites before
interfering with dynamic assembly of clathrin coats and the recycling of internalized receptor.

**Results**

**PtdIns(4,5)P₂ depletion blocks transferrin internalization**

We first confirmed that rapid PtdIns(4,5)P₂ hydrolysis can be induced in HeLa cells, because we used this cell system for the study. Within seconds after iRap addition, CF-Inp translocated from the cytosol to the PM, whereas the PH-domain of PLCδ, which binds to PtdIns(4,5)P₂ with high specificity and affinity (Stauffer et al., 1998), dissociated from the PM, indicating a loss of PM PtdIns(4,5)P₂ (Fig. 1A,B). iRap-induced PtdIns(4,5)P₂ depletion in HeLa cells caused a near complete inhibition of the internalization of Alexa-Fluor-594-conjugated transferrin, consistent with a proposed role of PtdIns(4,5)P₂ in clathrin-mediated endocytosis (Fig. 1C,D). In control experiments, cells expressing CF-Inp and treated with DMSO instead of iRap still internalized transferrin (Fig. 1C,D). Furthermore, iRap-induced translocation of CF-Inp(D281A) (Suh et al., 2006), a phosphatase-dead mutant of CF-Inp, had little, if any, effect on transferrin uptake (Fig. 1C,D), suggesting that the inhibition of transferrin uptake by CF-Inp translocation is due to its 5′ phosphatase activity rather than by a non-enzymatic effect of the inducible translocation system.

We then determined whether this block in clathrin-mediated transferrin receptor endocytosis reflects a general suppression of membrane internalization. We investigated the effect of rapid PtdIns(4,5)P₂ depletion on fluid-phase endocytosis, a clathrin-independent process (Nichols and Lippincott-Schwartz, 2001). iRap-induced translocation of CF-Inp had no significant effect on the uptake of fluorescein-Dextran (Mw 10,000), whereas it blocked the uptake of transferrin in the same cell (Fig. 1E,F). This indicates that the requirement of PM PtdIns(4,5)P₂ is selective for clathrin-mediated endocytosis over fluid-phase endocytosis.

**PtdIns(4,5)P₂ depletion increases transferrin receptor levels at the PM**

Recent studies suggest that PtdIns(4,5)P₂ is involved not only in endocytosis but also in the recycling of endocytosed cargoes back to the PM (Cremona and De Camilli, 2001). For example, deletion of PIPKγ, the major PtdIns(4,5)P₂-synthesizing enzyme in the brain, leads to defects in synaptic vesicle recycling (Di Paolo et al., 2004). In non-neuronal cells, the involvement of PtdIns(4,5)P₂ in the endosomal recycling pathway has been implicated from...
experiments in which the 5-phosphatase domain of synaptojanin was overexpressed, which in turn reduced the recycling rate of transferrin receptors (Kim et al., 2006). Although these studies suggest that prolonged changes in PtdIns(4,5)P₂ levels cause defects in endosomal recycling, it cannot be excluded that the observed effects on recycling are caused by indirect regulatory mechanisms or defects occurring elsewhere, not at the PM.

We tested the role of PM PtdIns(4,5)P₂ in the recycling process by reducing PM PtdIns(4,5)P₂ levels after having loaded the endocytic compartments with fluorescently labeled transferrin. We incubated HeLa cells for 20 minutes with Alexa-Fluor-594-conjugated transferrin before inducing PM PtdIns(4,5)P₂ hydrolysis. Recycling of internalized transferrin receptors was monitored by the loss of internal Alexa-Fluor-594-conjugated transferrin fluorescence as the ligand-receptor complex returned to the cell surface. PM PtdIns(4,5)P₂ hydrolysis following transferrin uptake caused a partial block of the reinsertion of Alexa-Fluor-594-conjugated transferrin fluorescence (Fig. 2A,B). A fraction of Alexa-Fluor-594-conjugated transferrin fluorescence appeared to accumulate both in endosomes and near the PM, suggesting that receptor-containing vesicles are less effective in fusing with the PM (Fig. 2A). In control experiments, PM translocation of the CF-Inp(D281A) phosphatase-dead mutant had no effect on recycling.

To assess the relative effect of PtdIns(4,5)P₂ hydrolysis on endocytosis versus that on recycling, we monitored the amount of transferrin receptors expressed at the cell surface. Using an antibody against the extracellular domain of the transferrin receptor, we found a 50% increase in the amount of receptor expressed at the PM after

![Image](image.png)

**Fig. 2.** PM PtdIns(4,5)P₂ hydrolysis partially inhibits transferrin receptor recycling to the surface while fully suppressing AP-2α PM localization and targeting of transferrin receptor (TfR) to endocytic sites. (A,B) Rapid PtdIns(4,5)P₂ hydrolysis causes partial inhibition of the reinsertion of internalized transferrin receptors back to the cell surface. Cells expressing Lyn-FRB and either CF-Inp or CF-Inp(D281A) were treated with Alexa-Fluor-594-conjugated transferrin for 20 minutes, then kept in Alexa-Fluor-594-conjugated-transferrin-free media for an additional 5, 15 or 30 minutes with iRap or DMSO. (A) Internal Alexa-Fluor-594-conjugated transferrin remaining in CF-Inp-translocated cells after 15 minutes of recycling. Arrows point to Lyn-FRB- and CF-Inp-expressing cells. Scale bars: 10 μm. (B) Time course of internal transferrin loss. Intracellular fluorescence intensity was normalized to that measured in cells expressing Lyn-FRB and CF-Inp(D281A) that were fixed immediately after Alexa-Fluor-594-conjugated-transferrin uptake without recycling (significance of difference P<7×10⁻¹¹ at the 15-minute time point). Error bars represent s.e.m. (C) Rapid PtdIns(4,5)P₂ hydrolysis increases the amount of transferrin receptors expressed at the PM, suggesting that PtdIns(4,5)P₂ regulates endocytosis significantly more than it does recycling. Cells expressing Lyn-FRB and CF-Inp were treated with iRap or DMSO for 15 or 30 minutes, then stained with transferrin receptor antibody to label surface transferrin receptors. Receptor expression is normalized to the fluorescence intensity measured in cells without iRap or DMSO treatment. Error bars represent s.e.m. (D) Antibody against AP-2α shows that PtdIns(4,5)P₂ depletion causes a dissociation of AP-2α puncta from the PM. Cells expressing Lyn-FRB and CF-Inp were treated with Alexa-Fluor-594-conjugated transferrin for 5 minutes following a 1-minute treatment with iRap. Dashed area indicates the location of Lyn-FRB- and CF-Inp-expressing cells. Scale bars: 10 μm. (E) Quantitative analysis of the PM dissociation of AP-2α using ratio imaging of the fluorescence intensity near the PM versus that of the midsection. Error bars represent s.e.m. (F) TIRF imaging of AP-2-μ2-YFP fluorescence at the PM better resolves the rapid loss of AP-2-μ2-YFP from the PM upon iRap-induced translocation of CF-Inp. Scale bars: 5 μm. (G-I) The AP-2-adaptor-mediated clustering of transferrin receptors is also lost upon PtdIns(4,5)P₂ hydrolysis. Cells expressing Lyn-FRB and either CF-Inp or CF-Inp(D281A) were treated with either DMSO or iRap for 5 minutes, then incubated with Alexa-Fluor-594-conjugated transferrin at 4°C to label transferrin receptors at the surface. Representative images are shown in G. Arrows point to Lyn-FRB- and CF-Inp-expressing cells. Scale bar: 10 μm. (H) Linescan of transferrin intensity from broken lines drawn in G reveals the loss of sharp peaks of transferrin intensity upon PtdIns(4,5)P₂ hydrolysis. (I) Mean of the standard deviations of transferrin intensity calculated from each linescan collected. Error bars represent s.e.m.
15 minutes of PtdIns(4,5)P₂ hydrolysis (Fig. 2C). There was no further increase in antibody fluorescence intensity after 30 minutes of hydrolysis, indicating that the recycling was blocked after 15 minutes. Assuming that synthesis of new receptors is negligible within the time-scale of minutes, the concentration of surface receptors is a direct function of the ratio between the endocytosis and recycling rates. Thus, the net increase in the level of transferrin receptor at the PM upon PtdIns(4,5)P₂ hydrolysis suggests that endocytosis and recycling have different sensitivities to PtdIns(4,5)P₂ reduction. PtdIns(4,5)P₂ can therefore directly regulate the surface expression of transferrin receptor by first suppressing endocytosis before interfering with the recycling process.

PtdIns(4,5)P₂ depletion dissociates AP-2 from the PM

Because PtdIns(4,5)P₂ has an indispensable role for transferrin receptor endocytosis, we focused our investigation on the molecular mechanism by which PtdIns(4,5)P₂ regulates this process. One proposed role of PM PtdIns(4,5)P₂ in clathrin-mediated endocytosis is in the recruitment of endocytic components to sites of endocytosis. Previous studies suggest that proteins important for the assembly of clathrin-coated vesicles bind to PtdIns(4,5)P₂ preferentially over other phosphoinositides, and local increases in PtdIns(4,5)P₂ serve as a key step in promoting receptor assembly in clathrin-coated pits (Krauss et al., 2003; Doughman et al., 2003). An important mediator of this assembly process is AP-2, which binds to various endocytosed receptors, clathrin and clathrin-coated-vesicle-associated proteins (Owen et al., 2004). Previous studies indicate that PtdIns(4,5)P₂ plays a role in the recruitment of AP-2 to endocytic sites at the PM. Mutant AP-2 with a deletion in the putative lipid-binding domain fail to localize to clathrin-coated pits (Gaidarov and Keen, 1999). In vitro studies suggest that PtdIns(4,5)P₂ is required for AP-2 to interact with the endocytic cargo (Honing et al., 2005). Furthermore, knockdown of phosphatidylinositol phosphate 5-kinase β, an enzyme that produces PtdIns(4,5)P₂, leads to a reduction of membrane-localized AP-2 (Padron et al., 2003). Rapid depletion of PM PtdIns(4,5)P₂ with 1-butanol or another inducible 5'-phosphatase translocation strategy was also shown to lead to a dissociation of AP-2 from the PM (Bocourt et al., 2006; Zoncu et al., 2007).

We used the PtdIns(4,5)P₂-specific Inp54p PM translocation system to confirm such a role of PtdIns(4,5)P₂ in AP-2 PM targeting using immunofluorescence and live total internal reflectance fluorescence (TIRF) microscopy. The distribution of AP-2α immunofluorescence observed in PtdIns(4,5)P₂-depleted cells was diffuse and cytosolic, in contrast to the punctate distribution near the PM observed in non-hydrolyzed control cells (Fig. 2D). The ratio of the average fluorescence intensity of AP-2α near the PM versus that in the midsection of the cell provides a quantitative measure for the loss of PM association of AP-2α upon PtdIns(4,5)P₂ hydrolysis (Fig. 2E). We further confirmed this effect using live TIRF imaging of YFP-tagged AP-2 μ2, a different component of the AP-2 adaptor complex. PtdIns(4,5)P₂ hydrolysis caused a rapid dissociation of AP-2-μ2–YFP from the PM within seconds, and a near complete loss by ~3-4 minutes (Fig. 2F). This finding that PtdIns(4,5)P₂ reduction leads to dissociation of AP-2 from the PM in less than 15 seconds supports the hypothesis that PtdIns(4,5)P₂ is directly required for targeting of AP-2 to the PM.

PtdIns(4,5)P₂ depletion prevents transferrin receptor clustering

Because AP-2 serves as an adaptor that directs receptors to clathrin-coated pits, we tested the effect of PtdIns(4,5)P₂ hydrolysis on transferrin receptor clustering. To visualize the localization of transferrin receptors at the PM, we incubated cells with Alexa-Fluor-594-conjugated transferrin at 4°C to label surface transferrin receptors. At this temperature, internalization of the ligand-receptor complex is inhibited. In PtdIns(4,5)P₂-hydrolyzed cells, we observed a uniform distribution of transferrin receptor compared with the punctate pattern observed in control cells (Fig. 2G). This effect was analyzed quantitatively using linescan analysis. Transferrin fluorescence intensity linescans showed sharp peaks in DMSO-treated control cells, reflecting the clustered distribution of LDL versus that in the midsection of the cell provides a quantitative measure for the loss of PM association of AP-2α upon PtdIns(4,5)P₂ hydrolysis (Fig. 2E). We further confirmed this effect using live TIRF imaging of YFP-tagged AP-2 μ2, a different component of the AP-2 adaptor complex. PtdIns(4,5)P₂ hydrolysis caused a rapid dissociation of AP-2-μ2–YFP from the PM within seconds, and a near complete loss by ~3-4 minutes (Fig. 2F). This finding that PtdIns(4,5)P₂ reduction leads to dissociation of AP-2 from the PM in less than 15 seconds supports the hypothesis that PtdIns(4,5)P₂ is directly required for targeting of AP-2 to the PM.

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Fig. 3. PM PtdIns(4,5)P₂ hydrolysis significantly suppresses LDL receptor endocytosis and weakly suppresses PM localization of epsin and CALM adaptors. (A,B) Following treatment with either DMSO or iRap, cells expressing Lyn-FRB and CF-Inp were simultaneously assayed for internalization of Dil-labeled LDL and Alexa-Fluor-488-conjugated transferrin. (A) Representative image of CF-Inp-expressing cells treated with iRap and fixed after transferrin and LDL uptake, showing suppression of the internalization of both receptors in the same cells. Dashed area indicates the location of Lyn-FRB- and CF-Inp-expressing cells. Scale bars: 10 μm. (B) Quantification of transferrin and LDL uptake. For each tracer, the internalization of the ligand-receptor complex is inhibited. In PtdIns(4,5)P₂-hydrolyzed cells, we observed a uniform distribution of transferrin receptor compared with the punctate pattern observed in control cells (Fig. 2G). This effect was analyzed quantitatively using linescan analysis. Transferrin fluorescence intensity linescans showed sharp peaks in DMSO-treated control cells, reflecting the clustered distribution of LDL versus that in the midsection of the cell provides a quantitative measure for the loss of PM association of AP-2α upon PtdIns(4,5)P₂ hydrolysis (Fig. 2E). We further confirmed this effect using live TIRF imaging of YFP-tagged AP-2 μ2, a different component of the AP-2 adaptor complex. PtdIns(4,5)P₂ hydrolysis caused a rapid dissociation of AP-2-μ2–YFP from the PM within seconds, and a near complete loss by ~3-4 minutes (Fig. 2F). This finding that PtdIns(4,5)P₂ reduction leads to dissociation of AP-2 from the PM in less than 15 seconds supports the hypothesis that PtdIns(4,5)P₂ is directly required for targeting of AP-2 to the PM.
transferrin receptors in clathrin-coated pits (Fig. 2H). Linescans across PtdIns(4,5)P_2-hydrolyzed cells lacked these peaks, reflecting a uniform distribution of transferrin receptors (Fig. 2H). We also calculated the standard deviation of transferrin receptor intensity values within each linescan using normalized fluorescence. Standard-deviation values of transferrin intensity in linescans across PtdIns(4,5)P_2-hydrolyzed cells were markedly smaller than those measured in control cells (Fig. 2I). Thus, PtdIns(4,5)P_2 is required not only for targeting AP-2 to the PM but also for the clustering of transferrin receptors into clathrin-coated pits. These findings support a model in which PtdIns(4,5)P_2 targets AP-2 to the PM and that PM-localized AP-2 is required to direct transferrin receptors to clathrin-coated pits.

**PtdIns(4,5)P_2 regulates LDL receptor endocytosis**

We tested whether PtdIns(4,5)P_2 has also a role in regulating the endocytosis of low density lipoprotein (LDL) receptors and endocytosis of the endocytic coat components CALM and epsin. Even though LDL receptors are mostly endocytosed by an AP-2-independent endocytic route, we found that its endocytosis was also suppressed by PtdIns(4,5)P_2 hydrolysis (Fig. 3A). A comparison of the relative suppression of endocytosis of transferrin receptors versus LDL receptors is shown in Fig. 3B. Furthermore, antibodies against the alternative adaptor proteins epsin and CALM revealed a partial loss from the PM in PtdIns(4,5)P_2-depleted cells (Fig. 3C). This argues that PtdIns(4,5)P_2 regulates the endocytosis of a range of receptors, possibly by regulating, with different efficiency, different endocytic adaptors.

**Assembly of PM clathrin is only weakly affected by PtdIns(4,5)P_2 depletion**

In addition to its role in targeting receptors to coated pits, AP-2 has also been proposed to have a role in the assembly of clathrin coats at the PM (Gallusser and Kirchhausen, 1993; Shih et al., 1995; Brodsky et al., 2001). We tested the effect of reducing PtdIns(4,5)P_2 concentration on clathrin-coat assembly and disassembly at the PM using live TIRF imaging of YFP-tagged clathrin light chain (CLC). We added Alexa-Fluor-594-conjugated transferrin at the same time as iRap was added, and analyzed those cells in which transferrin uptake was significantly reduced. Surprisingly, iRap-induced translocation of CFlInp led to only an approximately 20% reduction in the average intensity of clathrin puncta detected at the PM, suggesting that much of the clathrin remains clustered at the PM when PtdIns(4,5)P_2-levels were reduced after translocation of Inp54 (Fig. 4A,B, supplementary material Fig. S1).

Under these conditions, reduction in the number of detectable AP-2-μ2 puncta was more than fourfold stronger compared to that of clathrin puncta (Fig. 4C). Dynamic formation of new clathrin puncta was also still observed after iRap addition (supplementary material Movie 1). Furthermore, we did not observe a statistically significant change in the average lifetime of clathrin puncta upon hydrolysis of PtdIns(4,5)P_2 (52±3 seconds before iRap treatment, 47±3 seconds with iRap treatment) (Fig. 4D).

An earlier study reported that PtdIns(4,5)P_2 depletion with ionomycin, a strong activator of phospholipase C, or PM translocation of human 5′ phosphatase nearly completely blocks clathrin assembly (Zoncu et al., 2007). We confirmed their findings that addition of ionomycin suppresses dynamic clathrin assembly (supplementary material Movie 1). A plausible explanation for the discrepancy between their results and ours is that experimentally observed receptor-mediated reductions in PtdIns(4,5)P_2 concentrations are believed to be graded and typically incomplete (i.e. Stauffer et al., 1998), and an important
issue for understanding PtdIns(4,5)P2 signaling is whether different PtdIns(4,5)P2-regulated processes have different sensitivities to PtdIns(4,5)P2. Our study argues that physiological reductions in PtdIns(4,5)P2 primarily act on adaptor proteins such as AP-2 and thereby reduce the targeting of cargo to endocytic sites, whereas supramaximal reductions in PtdIns(4,5)P2 are needed to interfere with clathrin assembly or with receptor recycling back to the surface.

**Conclusion**

We conclude that PtdIns(4,5)P2 is required for transferrin receptor endocytosis, but not for fluid-phase endocytosis. Furthermore, although PtdIns(4,5)P2 plays a vital role in endocytosis, reductions in PtdIns(4,5)P2 have less of an effect on receptor recycling back to the surface. This differential role of PtdIns(4,5)P2 in endocytosis versus recycling leads to an increase in the amount of transferrin receptor in the PM following PtdIns(4,5)P2 reduction. Furthermore, our results suggest that a primary role of physiological decreases in PtdIns(4,5)P2 for endocytosis is a strong PM dissociation of adaptor complex AP-2, and a weaker PM dissociation of the adaptor proteins ephrin and CALM. This is consistent with a model in which receptor-mediated increases or decreases in the concentration of PtdIns(4,5)P2 control the PM localization of endocytic adaptors and thereby enhance or reduce the adaptor-mediated delivery of receptor cargo to endocytic sites. Based on our findings, the dynamic assembly of clathrin at the PM is much less affected by decreases in PtdIns(4,5)P2 concentration compared with the effects on AP-2, suggesting that physiological changes in PtdIns(4,5)P2 regulate adaptor-mediated targeting of cargo to endocytic sites rather than clathrin assembly.

**Materials and Methods**

**Cell culture, transfection and constructs**

HeLa cells were purchased from ATCC, and cultured in DMEM containing 10% FBS and 1% Penicillin-Streptomycin-Glutamine at 37°C at 10% CO2. Cells were plated on Costar 96-well flat-bottom plates for epifluorescence microscopy. For confocal microscopy, cells were plated on 8-well labtek coverslips (Nunc). 24 hours after plating, cells were transfected with FuGENE6 (Roche). All experiments were performed 18-20 hours after transfection. YFP-PLC-β1 (Stauffer et al., 1998), Lyn114 FRB, CFP-FKBP-Inp54p and CFP-FKBP-Inp54p (Suh et al., 2006) constructs are previously described. Construct requests should be made to T.I. FRB, CFP-FKBP-Inp54p and CFP-FKBP-Inp54p(D281A) (Suh et al., 2006). 200 μM iRap in DMEM-HEPES or 0.1% DMSO in DMEM-HEPES. 1 minute later, fluorescein-dextran (Invitrogen) and Alexa-Fluor-594-conjugated transferrin were added to the iRap or DMSO solution to a final concentration of 5 μg/ml Alexa-Fluor-594-conjugated transferrin. After 2 minutes of uptake, extracellular Alexa-Fluor-594-conjugated transferrin was removed by ice-cold acid solution containing 0.2 M acetic acid and 0.5 M NaCl, followed by DPBS neutralization. Cells were then incubated in 5 μM iRap or 0.1% DMSO in DMEM-HEPES containing 50 μg/ml holotransferrin (Sigma). Cells were fixed with 4% formaldehyde in PBS after 5, 15 or 30 minutes. For both the internalization and recycling assays, Alexa-Fluor-594-conjugated transferrin and CF-Inp were imaged on a 40x objective (Zeiss) on an automated epifluorescence microscope ImageXpress 5000A (Molecular Devices). Fluorescence intensities of single cells were measured with the ImageXpress analysis software.

**For dextran and transferrin internalization**, cells were first washed twice with DMEM and incubated with 5 μM iRap in DMEM-HEPES or 0.1% DMSO in DMEM-HEPES. 1 minute later, fluorescein-dextran (Invitrogen) and Alexa-Fluor-594-conjugated transferrin in DMEM-HEPES were added to the iRap or DMSO solution to a final concentration of 10 mg/ml fluorescein-dextran and 5 μg/ml Alexa-Fluor-594-conjugated transferrin. After 20 minutes of uptake, extracellular Alexa-Fluor-594-conjugated transferrin was removed by ice-cold acid solution containing 0.2 M acetic acid and 0.5 M NaCl, followed by neutralization with DPBS (GIBCO). Cells were then fixed with 4% formaldehyde in PBS after 5, 15 or 30 minutes. For both the internalization and recycling assays, Alexa-Fluor-594-conjugated transferrin and CF-Inp were imaged on a 40x objective (Zeiss) on an automated epifluorescence microscope ImageXpress 5000A (Molecular Devices). Fluorescence intensities of single cells were measured with the ImageXpress analysis software.

**Immunofluorescence**

To label surface transferrin receptors, anti-transferrin-receptor antibody staining was performed using live cells at 4°C to prevent receptor internalization. Cells were pre-incubated at 4°C in DMEM-HEPES for 10 minutes. Then, cells were immunostained with mouse anti-human CD71 in DMEM-HEPES with 1% BSA (1:1000; BD Pharmingen) for 45 minutes. Cells were then fixed for 10 minutes with 4% formaldehyde in PBS at room temperature and stained with primary anti-mouse IgG conjugated to Alexa-Fluor-568 or Alexa-Fluor-488-conjugated anti-mouse IgG in PBS with 1% BSA. Secondary antibody staining was performed with Alexa-Fluor-594-conjugated or Alexa-Fluor-488-conjugated goat anti-mouse IgG in PBS with 1% BSA (1:1000). For the transferrin receptor recycling assay, cells were first washed twice with DMEM and incubated with 5 μM iRap in DMEM-HEPES at 37°C, cells were washed with cold DMEM-HEPES and placed at 4°C for 10 minutes. Cells were then fixed with 4% formaldehyde in PBS at room temperature. Dil-LDL and transferrin internalization were performed similarly, with 5 μg/ml Dil-LDL and 5 μg/ml Alexa-Fluor-488-conjugated transferrin. Alexa-Fluor-594-conjugated transferrin, fluorescein-dextran, Dil-LDL and CF-Inp(D281A) fluorescence were imaged with a 63x objective (Zeiss) on the Bio-Rad Radiance 2100 Laser-scanning confocal system. Images were taken from the midsection of the cell. Parameters on camera and microscope were set so that no area was saturated, and the same setting was used to image all cells. Images were analyzed with MetaMorph 6.1 software (Universal Imaging). The background fluorescence of each image was subtracted before analysis.

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**Live microscopy**

CF-Inp and YFP-PLC-β1 translocation was monitored by live imaging with a 40x objective (Zeiss) on a spinning-disk confocal microscope (Nikon Wallac system). Images were analyzed with MetaMorph 6.1 software (Universal Imaging).

Objective-based TIRF imaging of YFP-CLC and AP-2-m2-YFP was performed with a NA 1.45 100x objective (Zeiss) on an inverted microscope (Zeiss) at 37°C. For each cell, YFP TIRF images were obtained every 1 seconds for 10 minutes, followed by addition of 5 μg/ml Alexa-Fluor-594-conjugated transferrin and 5 μM iRap. After addition, YFP TIRF images were obtained every 1 minutes for 10 minutes or until complete dissociation was observed. Alexa-Fluor-594-conjugated transferrin uptake was imaged using epifluorescence at the end of each experiment.

**Internalization and recycling assays**

All internalization and recycling assays were performed at 37°C. Prior to the transferrin-internalization assay, transferrin present in serum was removed by two washes with DMEM (with 25 mm HEPES, GIBCO). Cells were then incubated with 5 μM iRap in DMEM-HEPES or 0.1% DMSO in DMEM-HEPES. 1 minute later, Alexa-Fluor-594-conjugated transferrin (Invitrogen) in DMEM-HEPES was added to the iRap or DMSO solution to a final concentration of 5 μg/ml Alexa-Fluor-594-conjugated transferrin. After 2, 5, 15 or 30 minutes, extracellular Alexa-Fluor-594-conjugated transferrin was removed by ice-cold acid solution containing 0.2 M acetic acid and 0.5 M NaCl, followed by neutralization with DPBS (GIBCO). Cells were then fixed with 4% formaldehyde (Ted Pella) in PBS for 10 minutes at room temperature.

**For the transferrin receptor recycling assay, cells were first washed twice with DMEM and incubated with 5 μM iRap in DMEM-HEPES, then incubated with 5 μg/ml Alexa-Fluor-594-conjugated transferrin for 20 minutes. Extracellular Alexa-Fluor-594-conjugated transferrin was then removed with acid solution containing 0.2 M acetic acid and 0.5 M NaCl, followed by DPBS neutralization. Cells were then incubated in 5 μM iRap or 0.1% DMSO in DMEM-HEPES for 20 minutes. Extracellular Alexa-Fluor-594-conjugated transferrin was then removed with acid solution containing 0.2 M acetic acid and 0.5 M NaCl, followed by DPBS neutralization. Cells were then incubated in 5 μM iRap or 0.1% DMSO in DMEM-HEPES.**
Figs 1, 2 and Fig. 3B, we analyzed between 30 and 200 individual cells for each condition. For live TIRF imaging, we analyzed eight cells for AP-2-μ2–YFP and CFInp54 translocation, nine cells for YFP-CLC with CFInp54 translocation and three cells for YFP-CLC with CFInp(D281A) translocation.

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