

Role of activation of PIP5K γ 661 by AP-2 complex in synaptic vesicle endocytosis

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Synaptic vesicles (SVs) are retrieved by clathrin-mediated endocytosis at the nerve terminals. Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] drives this event by recruiting the components of the endocytic machinery. However, the molecular mechanisms that result in local generation of PI(4,5)P₂ remain unclear. We demonstrate here that AP-2 complex directly interacts with phosphatidylinositol 4-phosphate 5-kinase γ 661 (PIP5K γ 661), the major PI(4,5)P₂-producing enzyme in the brain. The β 2 subunit of AP-2 was found to bind to the C-terminal tail of PIP5K γ 661 and cause PIP5K γ 661 activation. The interaction is regulated by PIP5K γ 661 dephosphorylation, which is triggered by depolarization in mouse hippocampal neurons. Finally, overexpression of the PIP5K γ 661 C-terminal region in hippocampal neurons suppresses depolarization-dependent SV endocytosis. These findings provide evidence for the molecular mechanism through which PIP5K γ 661 locally generates PI(4,5)P₂ in hippocampal neurons and suggest a model in which the interaction trigger SV endocytosis.

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Introduction

Neurons communicate at synapses via neurotransmitters released from synaptic vesicles (SVs) that have fused with the presynaptic plasma membrane. After the release of neurotransmitters, the fused SVs are locally retrieved via endo-

cytosis, refilled with neurotransmitters and reutilized for subsequent rounds of neurotransmitter release at the active nerve terminal (Ryan *et al.*, 1993). Thus, efficient SV endocytosis is a critical step in maintaining synaptic activity. This step is mediated by the clathrin-dependent pathway (Murthy and De Camilli, 2003; Sudhof, 2004), although a second mechanism, referred to as 'kiss-and-run', may also play a role in rapid endocytosis at the synapses (Aravanis *et al.*, 2003).

In the early steps of clathrin-mediated endocytosis, components of the endocytic machinery, such as clathrin, the adaptor protein complex AP-2, amphiphysin, epsin and AP-180, assemble at the endocytic zones. Formation of the assembly is regulated, at least in part, by dephosphorylation of several components of the endocytic machinery, including amphiphysin, AP-180 and dynamin, which are referred to as dephosphins (Slepnev *et al.*, 1998; Cousin *et al.*, 2001). Dephosphins are constitutively phosphorylated by the neuron-specific dephosphin kinase Cdk5 in neurons at rest (Tan *et al.*, 2003; Tomizawa *et al.*, 2003). Upon excitation of the presynaptic terminal, the dephosphins become dephosphorylated by the Ca²⁺-dependent phosphatase calcineurin, which in turn triggers their interaction with the other components of the endocytic machinery (Cousin and Robinson, 2001).

Increasing evidence indicates that the pleiotropic membrane phospholipid, phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], interacts with and recruits several components of the endocytic machinery to endocytic hot spots to induce clathrin-mediated endocytosis (Gaidarov and Keen, 1999; Ford *et al.*, 2001; Itoh *et al.*, 2001; Collins *et al.*, 2002; Rohde *et al.*, 2002). In mammalian cells, PI(4,5)P₂ is produced predominantly by phosphatidylinositol-4-phosphate-5-kinase (PIP5K) through phosphorylation of phosphatidylinositol-4-phosphate PI(4)P at the D-5 position of the inositol ring (Ishihara *et al.*, 1996; Loijens *et al.*, 1996). The PIP5K gene products consist of three isozymes, α , β and γ (Ishihara *et al.*, 1998), and the γ isozyme has three splicing variants: PIP5K γ 635, which encode 635 amino-acid residues; PIP5K γ 661, which contains an additional 26 amino acids at the C-terminus; and PIP5K γ 687, which contains an additional 26 amino acids inserted before the C-terminal 26 amino-acid residues of PIP5K γ 661 (Giudici *et al.*, 2004). PIP5K γ , especially PIP5K γ 661, is highly expressed in the brain and concentrates at the neuronal synapses (Wenk *et al.*, 2001; Akiba *et al.*, 2002), whereas PIP5K α and β are expressed ubiquitously (Ishihara *et al.*, 1996, 1998; Loijens *et al.*, 1996). These reports, taken together with the fact that the PIP5K product PI(4,5)P₂ recruits several components of the clathrin-coated complex, imply that PIP5K has a crucial role in SV recycling, and this was confirmed by targeting the PIP5K γ gene (Di Paolo *et al.*, 2004).

A key question raised by this and other studies, although concerns the molecular mechanisms that direct PIP5K γ to produce PI(4,5)P₂ at endocytic hot spots of the presynaptic

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plasmalemma. If PIP5K γ interacts with an endocytic protein(s), PI(4,5)P₂ can be produced efficiently within the limited region of the endocytic hot spot, and thus lead to the assembly of PI(4,5)P₂-binding components of the endocytic machinery precisely at the endocytic hot spots, triggering efficient endocytosis of SVs. To address this issue, we have investigated whether PIP5K γ interacts with endocytic proteins of the clathrin-coated complex. We present evidence that neuronal depolarization results in dephosphorylation of PIP5K γ 661, leading to its activation via interaction with AP-2 at the nerve terminus, where it then triggers clathrin-mediated endocytosis of SVs.

Results

PIP5K γ interacts with AP-2 complex

To investigate whether PIP5K γ interacts with endocytic protein at presynapses, endogenous PIP5K γ in postnatal day (P)5 mouse brain was immunoprecipitated with an anti-PIP5K γ antibody and the co-immunoprecipitating proteins analyzed by Western blotting with antibodies specific to components of the endocytic machinery. α and β adaptin, which are components of the AP-2 complex, were found to co-immunoprecipitate with PIP5K γ , but not other endocytic proteins examined, including γ adaptin, which is an AP-1 complex component, clathrin heavy chain, amphiphysin and AP-180 (Figure 1A). Thus, PIP5K γ and AP-2 interact endogenously and in a relatively specific manner.

The AP-2 complex consists of four subunits, α , β , μ 2 and σ 2 (Virshup and Bennett, 1988; Matsui and Kirchhausen, 1990), as schematically depicted in Figure 1B. To identify the AP-2 subunit interacting with PIP5K γ , GST- or Flag-tagged AP-2 subunits purified from *Escherichia coli* were incubated with lysates of HEK293T cells overexpressing Myc-PIP5K γ 661, and then pulled down with glutathione-Sepharose or anti-Flag M2 affinity gel (Figure 1C). In this experiment, PIP5K γ 661 was employed because this splicing variant is the one most abundantly expressed in the brain (Wenk *et al*, 2001; Akiba *et al*, 2002). It is clear from the results shown in Figure 1C that β 2 adaptin is the component responsible for the PIP5K γ 661 interaction. Intriguingly, the PIP5K γ 661 expressed in HEK293T cells migrated as a doublet on the SDS-PAGE, but only the lower band was pulled down by β 2 adaptin.

To investigate the specificity of the β adaptin-PIP5K γ 661 interaction, interactions with all four of β adaptin isoforms were examined (Figure 1D). We found that in addition to PIP5K γ 661 interacting with β 2 adaptin, an interaction was also observed with the AP-1 complex component β 1 adaptin, which is \sim 80% identical to β 2 adaptin. As interaction of the endogenous AP-1 complex with endogenous PIP5K γ was not observed (Figure 1A), the interaction of β 1 adaptin with PIP5K γ 661 in this less physiological *in vitro* system may reflect the relaxed stringency of this approach.

To determine the interaction site on β 2 adaptin, we generated and purified three bacterial GST fragments: an N-terminal region, which forms the AP-2 complex core; the Ear domain, which binds to other endocytic components; and the Hinge domain, which connects the Ear domain to the β 2 adaptin core region (Zaremba and Keen, 1985; Heuser and Keen, 1988; Kirchhausen *et al*, 1989) (shown schematically in

Figure 1B). Pull-down assays using these fragments identified the Ear domain as the PIP5K γ 661-interacting region (Figure 1E). Again, the Ear domain appeared to interact only with the lower PIP5K γ 661 band.

AP-2 plays a role as an 'adaptor' via its Ear and Hinge domains in the recruitment of clathrin and several other accessory proteins to the endocytic zone (Shih *et al*, 1995; Praefcke *et al*, 2004). As lysates of HEK293T cells were used as a source of PIP5K γ 661 for the experiments described above, we could not totally exclude the possibility that the interaction might be indirect. This is not likely, however, as Flag-PIP5K γ 661 purified from *E. coli* interacted with purified bacterial GST- β 2 adaptin and its Ear domain in the *in vitro* assay system (Figure 1F). Thus, the interaction of PIP5K γ 661 with β 2 adaptin appears to be direct.

Interaction with β 2 adaptin is specific to PIP5K γ 661

Although PIP5K γ 661 is the most abundant isoform/splice variant expressed in the brain, PIP5K α , PIP5K β and multiple splice forms of PIP5K γ are also present. As well, PIP5Ks are thought to play roles at many subcellular locations in addition to endocytic initiation at the plasma membrane. These considerations raised the question of whether the β 2 adaptin-PI5PK interaction might be restricted to specific isozymes or splice variants. To address this point, GST- β 2 adaptin purified from *E. coli* was incubated with lysates of HEK293T cells expressing Flag-PIP5K isozymes and Flag-PIP5K γ splicing variants, which have the conserved kinase domain in their center and non-conserved regions in their N and C termini, as schematically depicted in Figure 2A, and the interacting proteins detected by GST pull-down (Figure 2B). We found that PIP5K γ 661 is the only isozyme/splice variant that interacts with β 2 adaptin. It was again observed that only the lower band of the PIP5K γ 661 doublet on SDS-PAGE gels interacted with β 2 adaptin.

As PIP5K γ 635, which lacks the C-terminal 26 amino acids of PIP5K γ 661, failed to interact with β 2 adaptin (Figure 2B), this suggested that the PIP5K γ 661 interaction site resides at its C-terminus. This was confirmed through the demonstration that the C-terminal region of PIP5K γ 661 (PIP5K γ 661C), but not that of PIP5K γ 635 (PIP5K γ 635C), co-precipitated with β 2 adaptin (Figure 2C), and similarly that the ability to co-precipitate chimeras of PIP5K β and PIP5K γ isozymes correlated with the C-terminal region being derived from PIP5K γ 661 (Figure 2D).

β 2 Adaptin activates PIP5K γ 661

To investigate the significance of the β 2 adaptin-PIP5K γ 661 interaction, PIP5K γ 661 and full-length or fragments of β 2 adaptin were expressed in and purified from HEK293T cells and *E. coli*, respectively, combined and assessed for PIP5K γ 661 kinase activity. Surprisingly, incubation with β 2 adaptin increased PIP5K γ 661 activity approximately eight-fold over the control (Figure 3A). When the lipid kinase-dead PIP5K γ 661 mutant (KD, D277A), in which Ala is substituted for the presumably critical Asp 277 in the putative ATP and PI(4)P binding site (Rao *et al*, 1998), was measured under the same conditions, β 2 adaptin did not elicit any increase in PIP5K activity (Figure 3B), eliminating the possibility of contamination of other lipid kinase(s) in the assay system. The β 2 adaptin Ear fragment, which had been found

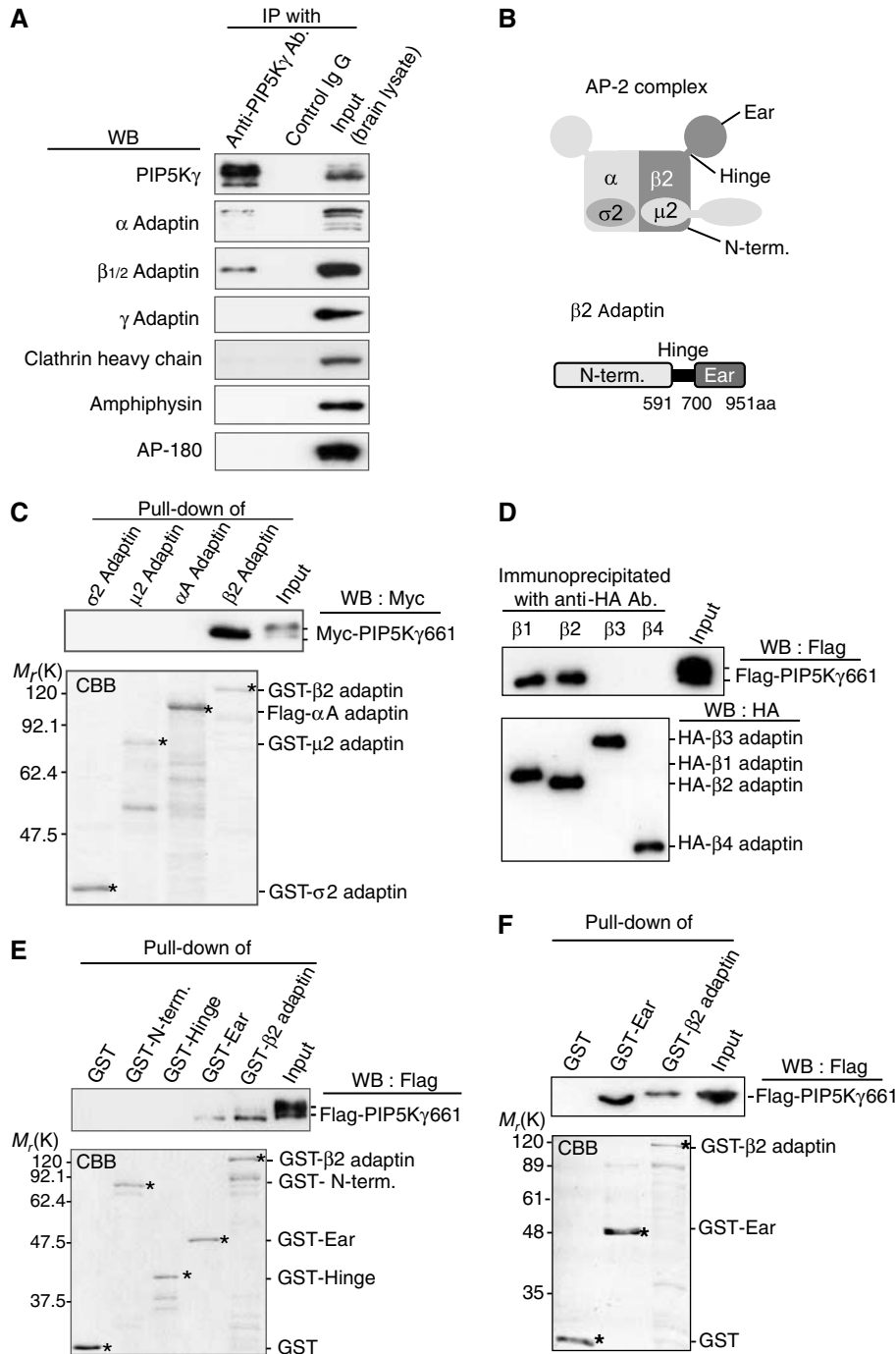


Figure 1 PIP5K γ 661 interacts with the AP-2 complex subunit β 2 adaptin. (A) Western blot of mouse brain lysate immunoprecipitated with polyclonal anti-PIP5K γ antibody. (B) Schematic representation of the AP-2 complex and the domain structure of its β 2 adaptin subunit. (C) GST- or Flag-tagged subunits of AP-2 purified from *E. coli* were incubated with lysates of HEK293T cells expressing Myc-PIP5K γ 661, pulled down and probed for co-precipitated Myc-PIP5K γ 661 using anti-Myc antibody. The lower panel (CBB) shows the input of the tagged subunits subjected to pull-down. (D) Immunoprecipitates of β 1–4 adaptins incubated with Flag-PIP5K γ 661-expressing HEK293T cell lysates. Co-immunoprecipitated Flag-PIP5K γ 661 was detected using anti-Flag M2 antibody. (E) Pull-downs of GST-tagged β 2 adaptin fragments incubated with Flag-PIP5K γ 661-expressing HEK293T lysates. Co-precipitated Flag-PIP5K γ 661 was detected using anti-Flag M2 antibody. The lower panel (CBB) displays the input protein fragments. (F) GST- β 2 adaptin and its Ear domain purified from *E. coli* were incubated with bacterially expressed and purified Flag-PIP5K γ 661, pulled down and probed for co-precipitated Flag-PIP5K γ 661 using anti-Flag M2 antibody. The lower panel (CBB) shows the input proteins. Asterisks, full-length input tagged proteins.

to be the binding domain for PIP5K γ 661, was confirmed to be responsible for PIP5K γ 661 activation, and did so in a dose-dependent manner (Figure 3A and C). Thus, AP-2 β 2 adaptin functions as a PIP5K γ 661 activator.

Phosphorylation of PIP5K γ 661 by Cdk5 interferes with the interaction with β 2 adaptin

As shown in Figures 1B, C and 2B, D, PIP5K γ 661 migrated as a doublet on SDS-PAGE and only the lower band appeared to

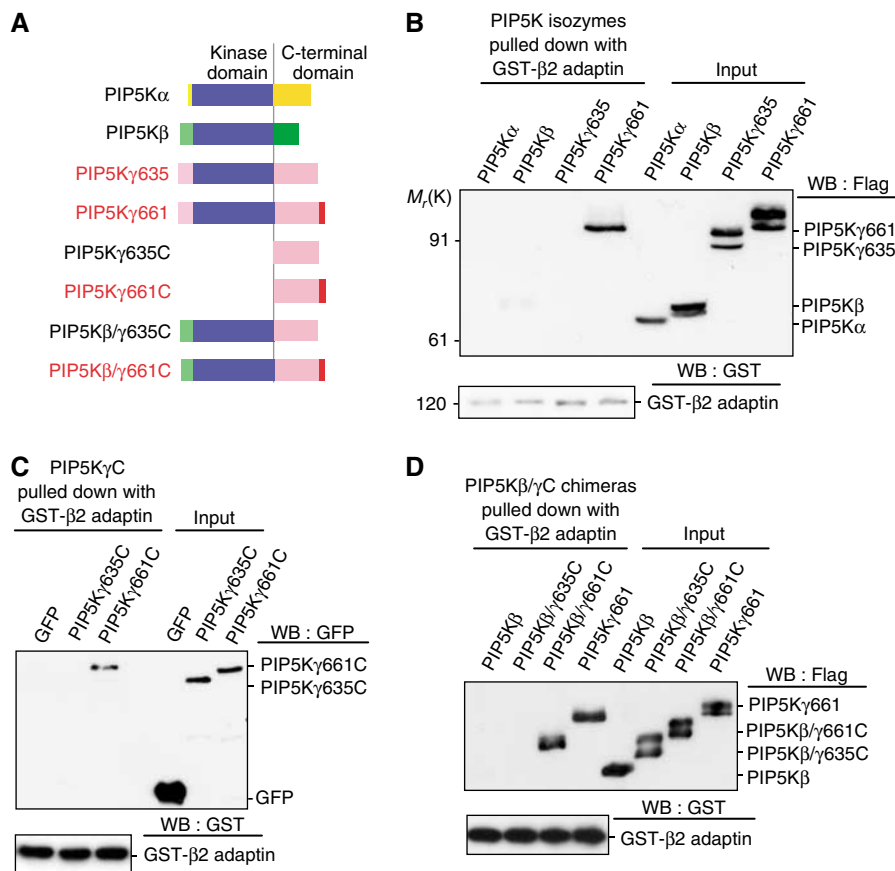


Figure 2 β 2 adaptin interacts specifically with PIP5K γ 661 through its C-terminus. (A) Schematic representation of PIP5K isozymes, PIP5K γ C-terminal regions (C) and PIP5K β/γ C chimeras used. (B–D) GST- β 2 adaptin purified from *E. coli* was pulled down after incubation with lysates from HEK293T cells expressing Flag-PIP5K isozymes (B), GFP-PIP5K γ Cs (C) and GFP-PIP5K β/γ C chimeras (D), which were detected using anti-Flag M2 and -GFP antibodies.

interact with β 2 adaptin. This observation suggested that PIP5K γ 661 might be variably phosphorylated and the interaction with AP-2 was dependent on the phosphorylation state. To explore this possibility, the PIP5K γ 661 was treated with the nonspecific phosphatase λ -PPase, which resulted in the disappearance of the upper band (Figure 4A). No effect was seen after treatment with the tyrosine-specific phosphatase YOP (Figure 4A); however, treatment with the Ser/Thr phosphatase PP1 eliminated the upper band and increased the efficiency of interaction with β 2 adaptin (Figure 4B). These results support the hypothesis that interaction with β 2 adaptin is regulated by dephosphorylation of PIP5K γ 661 on Ser/Thr residue(s).

It has been well documented that multiple endocytic components, collectively termed ‘dephosphins’, are constitutively phosphorylated by Cdk5 and their functions suppressed when neurons are in the resting state (Cousin and Robinson, 2001); thus, the above finding above suggested that PIP5K γ 661 might represent a new dephosphin. Within the C-terminal 26-amino-acid tail that mediates the interaction with β 2 adaptin, PIP5K γ 661 has a Cdk5 phosphorylation consensus site (Ser-Pro) (Figure 4C). To test the significance of this site, Cdk5 and its activator p35 were coexpressed with PIP5K γ 661 and β 2 adaptin in HEK293T cells, and phosphorylation of PIP5K γ 661 and interaction with β 2 adaptin were assessed. Coexpression of p35/Cdk5 drastically reduced both the dephosphorylated form of PIP5K γ 661 and the interaction

with β 2 adaptin (Figure 4D). To establish that Ser 645 is the site of phosphorylation in PIP5K γ 661 by Cdk5, a critical component for the interaction, we generated PIP5K γ 661 mutants that mimic the dephosphorylated and phosphorylated states, S645A and S645E, respectively. S645A, the dephosphorylation mimic, as well as wild-type PIP5K γ 661, could be pulled down with the GST-Ear domain of β 2 adaptin, whereas the pull down of S645E was largely eliminated (Figure 4E). We further confirmed the requirement of PIP5K γ 661 phosphorylation at Ser 645 for the interaction with β 2 adaptin using a mutant PIP5K γ 661 C-terminal fragment (PIP5K γ 661C), C-S645A. We reasoned that if C-S645A constitutively interacted with β 2 adaptin, it might be able to interfere with the interaction between endogenous PIP5K γ 661 and AP-2 complex, and thereby be a very useful tool to investigate the physiological relevance of their interaction. In contrast, the full-length PIP5K γ 661 mutant S645A might interfere with not only the interaction between these molecules, but also signaling through the small GTPase ARF6, which binds to PIP5K γ 661 and plays a role in the recruitment of clathrin/AP-2 to the presynaptic plasma membrane (see details in Discussion). Thus, C-S645A might be the better tool with which to investigate physiological significance of the interaction of these molecules.

Expression of p35/Cdk5, but not p35/kinase-dead Cdk5 (Cdk5 KD), inhibited interaction of wild-type PIP5K γ 661C with the β 2 adaptin Ear domain (Figure 4F). In contrast,

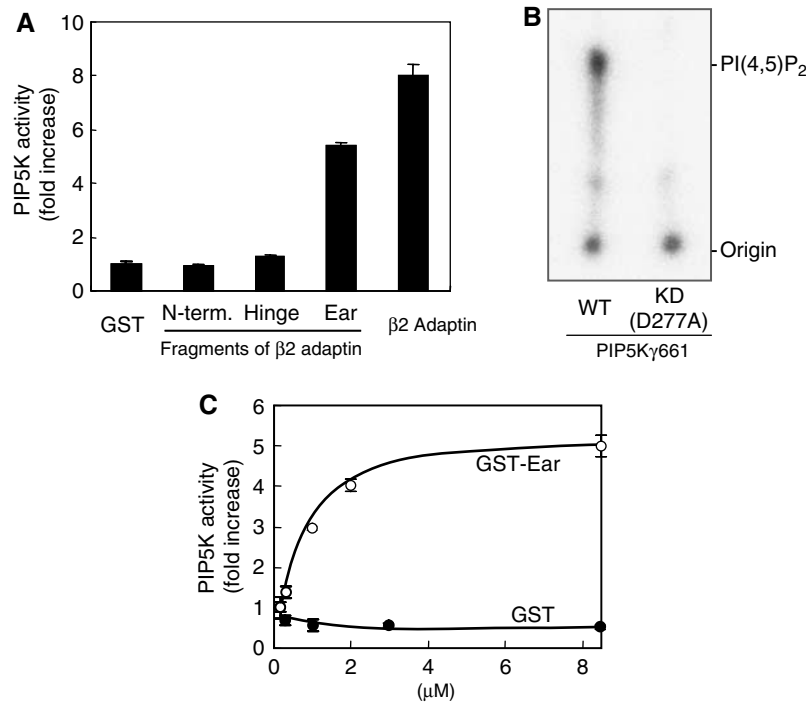


Figure 3 β 2 adaptin activates PIP5K γ 661. (A, B) Flag-PIP5K γ 661 and kinase-dead (KD) Flag-PIP5K γ 661 mutant D277A purified from HEK293T cells were incubated with GST- β 2 adaptin or GST fragments of β 2 adaptin purified from *E. coli*. [32 P]PI(4,5)P₂ produced by PIP5K γ 661s was determined as described in Materials and methods (A, C) or detected by autoradiography using thin-layer chromatography (B). Basal activity of PIP5K γ 661 measured in the presence of control GST in (A) was 0.51 ± 0.046 fmol/min/assay ($n = 6$). (C) Flag-PIP5K γ 661 was incubated with the indicated concentrations of GST-Ear domain or control GST, and [32 P]PI(4,5)P₂ production was assessed. Results in (A) and (C) are the mean \pm s.e.m., $n = 4$.

coexpression of p35/Cdk5 was unable to inhibit the interaction of C-S645A with the β 2 adaptin Ear domain. It is noteworthy that wild-type PIP5K γ 661C did not migrate as a doublet, whereas full-length PIP5K γ 661 did, suggesting that PIP5K γ 661 has multiple phosphorylation sites at the Ser/Thr residues for Cdk5 and other kinases that in total account for its altered mobility on SDS-PAGE. Nonetheless, taken together, these results clearly demonstrate that dephosphorylation of PIP5K γ 661 on Ser 645 is required for it to interact with the AP-2 complex. Moreover, this interaction is functionally significant, as the PIP5K activity of S645A is stimulated by β 2 adaptin, whereas S645E activity is not (Figure 4G).

PIP5K γ 661 interacts with β 2 adaptin when it becomes dephosphorylated upon depolarizing stimulation of mouse hippocampal neurons

Analysis of primary mouse hippocampal neurons prepared from embryonic day (E)17.5 mice and cultured for 3 weeks revealed that the expression level of PIP5K γ 661, which was initially undetectable, began to rise at day 10 and increased steadily thereafter (see Supplementary Figure S1). Expression of the short splice variant, PIP5K γ 635, remained undetectable through the culture period (see Supplementary Figure S1), indicating that PIP5K γ 661 is the predominant PIP5K isozyme in hippocampal neurons. This observation, taken together with the results described above, led us to speculate that PIP5K γ 661 should be constitutively phosphorylated by Cdk5 in the resting state and then become dephosphorylated and interact with AP-2 upon neuronal excitation. This hypothesis was explored using primary cultures of mouse hippocampal neurons. PIP5K γ 661 was in fact observed to

be predominantly phosphorylated in the resting state, and to become dephosphorylated upon depolarization triggered by high K⁺ stimulation (Figure 5A–C). Concomitantly, the interaction of PIP5K γ (possibly PIP5K γ 661) with β adaptin (possibly β 2 adaptin) increased in response to stimulation (Figure 5B). Dephosphorylation of PIP5K γ 661 appeared to be attributable to the action of the calcium-dependent phosphatase calcineurin, as calcineurin-specific inhibitor cyclosporin A (CysA) treatment of hippocampal neurons prevented the depolarization-dependent dephosphorylation of PIP5K γ 661 (Figure 5C). Furthermore, it was found that depolarizing stimulation of the hippocampal neurons increased the activity of the endogenous PIP5K γ (Figure 5D).

If the interaction of PIP5K γ 661 with β 2 adaptin and its subsequent activation upon a depolarizing stimulation is the mechanism through which PI(4,5)P₂ is produced at restricted areas of the presynaptic plasma membrane to induce SV endocytosis, one would expect that the interaction would take place at the presynapses. To address this point, intracellular localization of PI(4,5)P₂, β 2 adaptin and endogenous PIP5K γ 661 in hippocampal neurons was assessed by immunocytochemistry (the antibody used in this experiment does not discriminate between the PIP5K γ splice variants; however, PIP5K γ 661 is the predominant variant found in hippocampal neurons). In the resting state, PI(4,5)P₂ distributed diffusely in hippocampal neurons, but then shifted to presynaptic marker protein synaptotagmin I-positive punctate structures upon depolarizing stimulation (Figure 5E). This was also observed in hippocampal neuronal axons by live image analysis using a GFP-tagged PH domain of phospholipase C δ (GFP-PLC δ PH), which specifically binds to PI(4,5)P₂

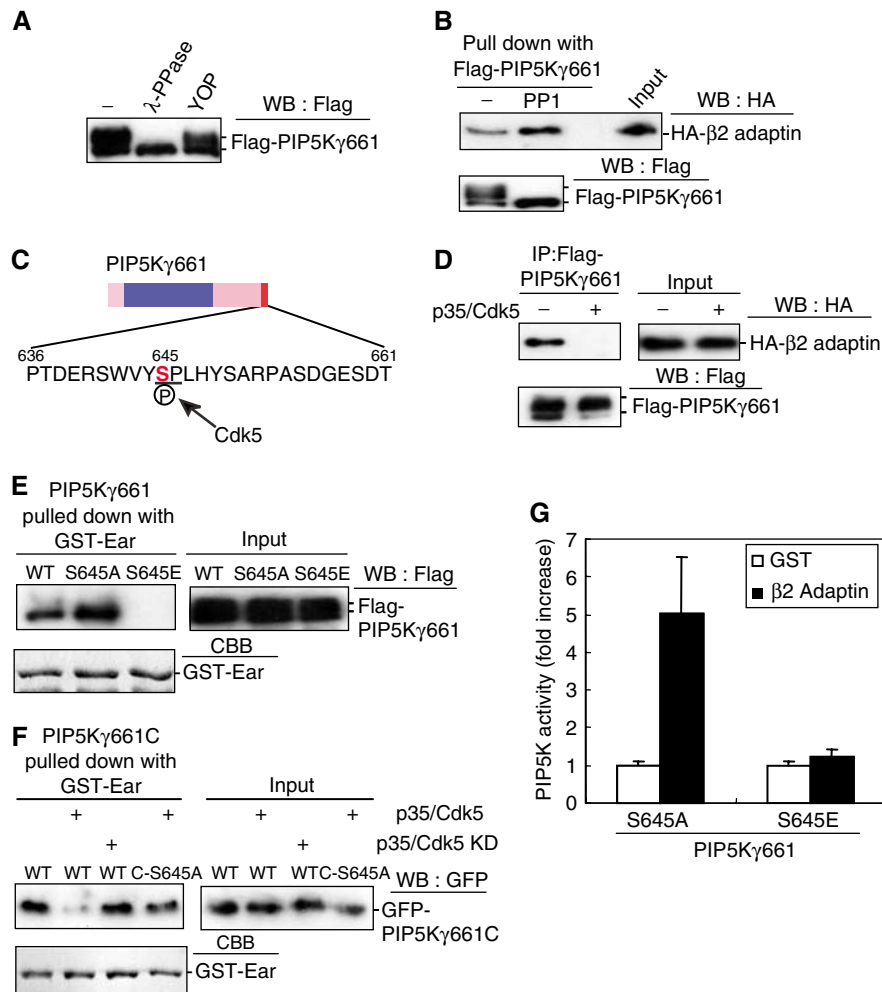


Figure 4 Dephosphorylation of PIP5K γ 661 on Ser 645 regulates its interaction with AP-2. (A) Flag-PIP5K γ 661 immunopurified from HEK293T cells was treated with λ -PPase and YOP, and its migration pattern in SDS-PAGE was assessed. (B) Immunopurified Flag-PIP5K γ 661 was treated with PP1, incubated with lysates of HEK293T cells expressing HA- β 2 adaptin, pulled down using anti-Flag M2 antibody and probed for co-precipitated HA- β 2 adaptin. (C) Amino-acid sequence of the C-terminal 26-amino-acid tail of PIP5K γ 661, which contains a possible phosphorylation site for Cdk5. (D) Immunoprecipitation of Flag-PIP5K γ 661 coexpressed with HA tagged β 2 adaptin and p35/Cdk5 in HEK293T cells. (E, F) GST-Ear domain of β 2 adaptin purified from *E. coli* was incubated with lysates of HEK293T cells expressing Flag-PIP5K γ 661 and its mutants S645A and S645E (E) and GFP-PIP5K γ 661C and its mutants C-S645A and C-S645E, with p35/Cdk5 or p35/Cdk5 KD (F). The GST-Ear domain was then pulled down; co-precipitated PIP5K γ 661s and its C-terminal fragments were detected using anti-Flag M2 and -GFP antibodies. (G) Flag-tagged mutants of PIP5K γ 661, S645A and S645E, purified from HEK293T cells were incubated with GST or GST- β 2 adaptin purified from *E. coli* and then PIP5K activity was measured as in Figure 3A. Results shown are the mean \pm s.e.m., $n = 4$.

(Figure 5F, upper panels). Live image analysis of GFP- β 2 adaptin revealed that, like PI(4,5)P $_2$, β 2 adaptin, which is distributed diffusely in the resting state, shifted to the punctate structures in response to depolarizing stimulation (Figure 5F, lower panels). These results support the notion described above. However, endogenous PIP5K γ (possible PIP5K γ 661) colocalized with the synaptotagmin I-positive punctate structures in axons even in the resting state, and its location did not change upon depolarizing stimulation (Figure 5G), suggesting that the localization of PIP5K γ 661 may be regulated by an unidentified molecule rather than AP-2 complex. This idea is supported by results showing that the localization of PIP5K γ 661 in hippocampal neurons was unaltered in cells in which endogenous β 2 adaptin had been knocked down by RNAi (see Supplementary Figure S4). These results, taken together, suggest that upon depolarizing stimulation, PIP5K γ 661 interacts with AP-2 at presynaptic

boutons, becomes activated and generates PI(4,5)P $_2$ at this restricted region of the presynapse plasma membrane.

PIP5K γ 661 C-terminal fragments, which interact with β 2-adaptin, inhibit SV endocytosis

The results described above raised the question of whether the interaction of PIP5K γ 661 with AP-2 at presynapses is critical for SV endocytosis. We explored this possibility by expressing C-terminal fragments of PIP5K γ 661, either wild-type or mutated at S645 to the dephosphomimetic form (C-S645A) or the phosphomimetic form (C-S645E). We hypothesized that if the dephosphorylation-dependent binding of endogenous PIP5K γ 661 to β 2 adaptin is critical for SV endocytosis, then different phenotypes should be observed with expression of these fragments; wild-type PIP5K γ 661C and C-S645A should be able to inhibit SV endocytosis induced by depolarizing stimulation owing to competitive inhibition

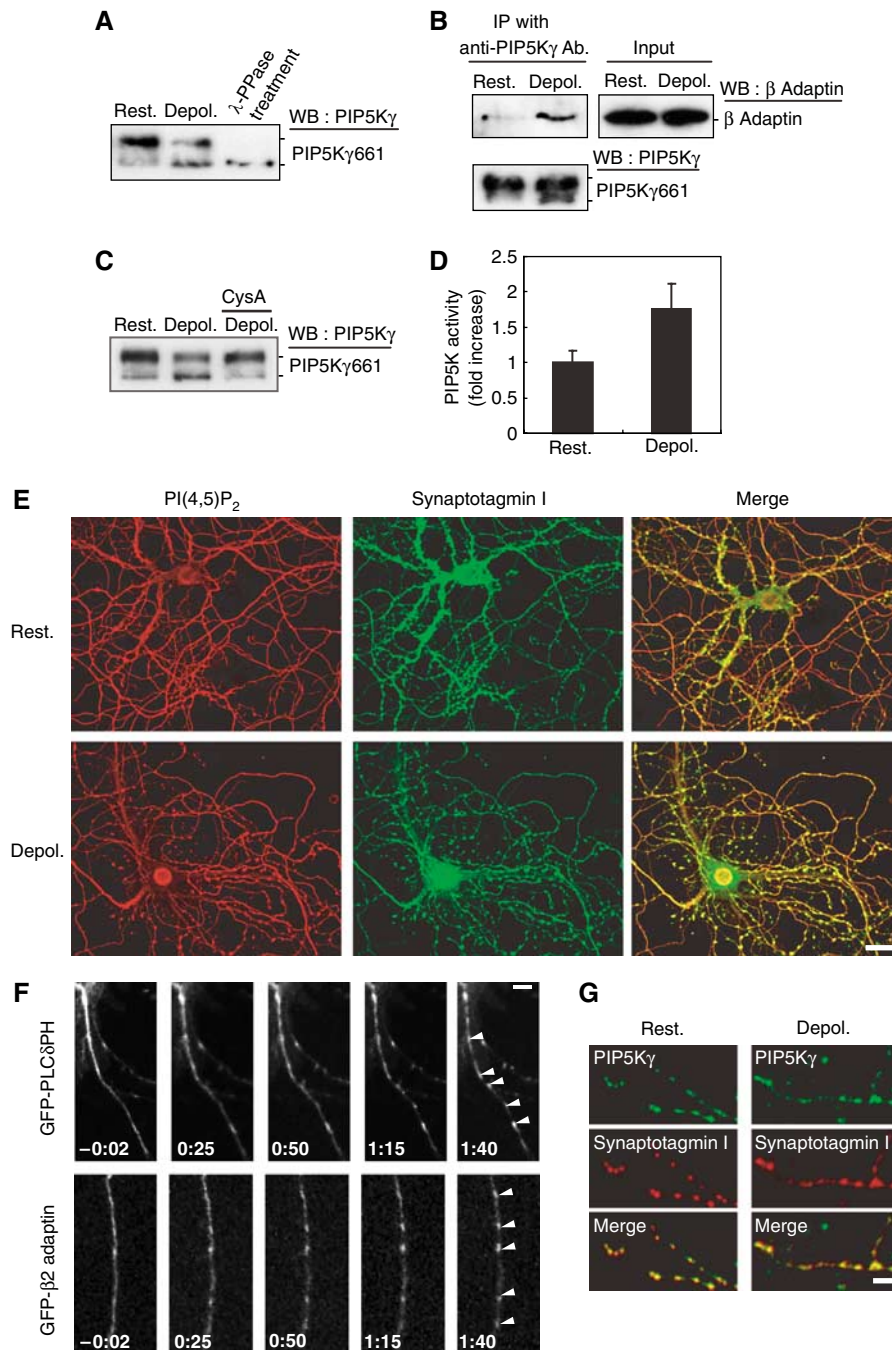


Figure 5 Interaction of PIP5K γ 661 with AP-2 is induced by dephosphorylation of PIP5K γ 661 as triggered by depolarizing stimulation of hippocampal neurons. (A) Mouse hippocampal neurons cultured for 19 days *in vitro* were stimulated with or without 45 mM KCl for 2 min and the phosphorylation state of PIP5K γ 661 was analyzed by Western blotting. The right lane shows a band of dephosphorylated PIP5K γ 661 prepared by treatment with λ -PPase. (B) Cultured hippocampal neurons were stimulated with KCl as in (A); AP-2 co-immunoprecipitating with PIP5K γ was detected using anti- β adaptin antibody. (C) Cultured hippocampal neurons were treated with 10 μ M cyclosporin A (CysA) for 30 min. After stimulation with 45 mM KCl, the phosphorylation state of PIP5K γ 661 was assessed by Western blotting. (D) Cultured hippocampal neurons were stimulated with or without KCl as in (A). PIP5K γ was then immunoprecipitated from the neurons and PIP5K activity was determined as described in Materials and methods. (E) Cultured hippocampal neurons were stimulated with or without 45 mM KCl, following which the subcellular localization of PI(4,5)P₂ and the presynaptic protein synaptotagmin I was visualized by immunostaining with specific antibodies. Scale bar, 20 μ m. (F) Cultured hippocampal neurons expressing GFP-PLC δ PH and GFP- β 2 adaptin were stimulated without or with 45 mM KCl, following which time-lapse images of expressed GFP proteins were collected at the indicated time intervals. Scale bar, 5 μ m. (G) Cultured hippocampal neurons were stimulated as in (E), following which the subcellular localizations of endogenous PIP5K γ and synaptotagmin I in axons were visualized by immunostaining with specific antibodies. Scale bar, 5 μ m.

of β 2 adaptin interaction with endogenous PIP5K γ 661, and C-S645E should not be able to bind or compete. When GFP-tagged wild-type PIP5K γ 661C was transiently expressed in hippocampal neurons using sindbis virus, structural synapses

formed, as assessed by immunostaining of synaptotagmin I (see Supplementary Figure S2), indicating that sindbis virus infection and overexpression of wild-type PIP5K γ 661C do not interfere with synapse formation. Under these conditions,

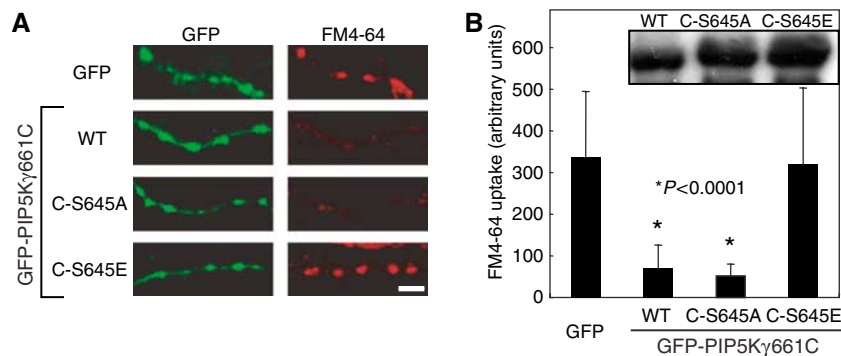


Figure 6 AP-2 complex-interacting PIP5K γ 661 C-terminal fragments interfere with SV endocytosis. (A) Primary cultured mouse hippocampal neurons were infected with sindbis pseudovirions to express GFP, GFP-PIP5K γ 661C or its mutants, C-S645A and C-S645E. After inducing depolarization with 45 mM KCl, the expressed proteins (green, left panels) and stimulated uptake of FM4-64 (red, right panels) were visualized. Scale bar, 5 μ m. (B) The total amount of stimulation-dependent FM4-64 accumulation was determined from the data (A) and represented as mean \pm s.e.m. Data are taken from 3–5 independent experiments ($n = 227$ nerve terminals for GFP control; $n = 112$ nerve terminals for GFP-PIP5K γ 661C WT; $n = 117$ nerve terminals for GFP-C-S645A; $n = 196$ nerve terminals for GFP-PIP5K γ 661C C-S645E). ‘Inset’ shows the expression levels of GFP-PIP5K γ 661C and its mutants in hippocampal neurons as analyzed by Western blots probed with anti-GFP antibody.

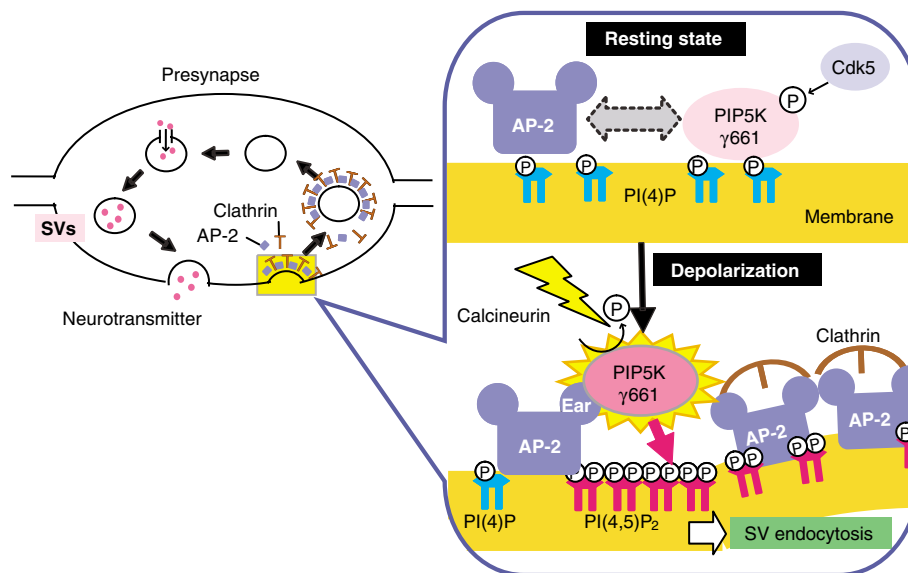


Figure 7 A novel model for the molecular mechanism through which SV endocytosis is induced. In the resting state, PIP5K γ 661 is constitutively phosphorylated by Cdk5 and its interaction with AP-2 is suppressed. Upon depolarization, PIP5K γ 661 becomes dephosphorylated, presumably by calcineurin, and then interacts with AP-2 in restricted regions of the presynaptic plasma membrane. The interaction of AP-2 stimulates PIP5K γ 661 kinase activity, resulting in local production of PI(4,5)P₂, recruitment of additional endocytic components and SV recycling.

transient expression of C-S645A, as well as the wild-type PIP5K γ 661C, significantly inhibited the depolarization-dependent SV endocytosis (Figure 6A and B). In contrast, expression of C-S645E was without any effect. These distinct effects of the PIP5K γ 661 C-terminal fragments were not due to differences in their expression levels (Figure 6B, inset). These findings demonstrate that the interaction between PIP5K γ 661 and β 2 adaptin, which is regulated by PIP5K γ 661 phosphorylation at Ser 645, occurs *in vivo* at the presynapse, and suggest that it triggers SV recycling.

Discussion

In the present study, we identify a new PIP5K γ 661-binding partner, the AP-2 adaptor complex, and show that it stimulates the PI(4,5)P₂-producing activity of PIP5K γ 661 at endo-

cytic hot spots. We also demonstrate that PIP5K γ 661 C-terminal fragments, which interact with AP-2 complex, interfere with depolarization-dependent SV endocytosis in mouse hippocampal neurons. The results obtained in this study lead us to propose a novel model for SV endocytosis (Figure 7): at the presynapse, PIP5K γ 661 is constitutively phosphorylated by Cdk5 in the resting state. Upon depolarization, it becomes dephosphorylated by calcineurin and then interacts with AP-2, resulting in activation of PIP5K γ 661 and production of PI(4,5)P₂ at endocytic hot spots, which in turn efficiently induces SV endocytosis by recruiting components of the clathrin-coated complex. This model provides insights into not only the activation mechanism of PIP5K γ 661, but also into the molecular mechanism through which SV endocytosis is triggered in restricted regions of the presynaptic plasma membrane.

Recently, Bairstow *et al* (2006); Krauss *et al* (2006) reported that the μ 2 subunit of AP-2 complex directly interacts with PIP5K γ 661, although the binding sites of PIP5K γ 661 reported are in conflict: Krauss *et al* reported the PIP5K γ kinase core domain as the interacting site, whereas Bairstow *et al* identified the 26-amino-acid C-terminal tail of PIP5K γ 661 as the binding site. In our experiment, shown in Figure 1C, where the AP-2 complex subunit interaction with PIP5K γ 661 was investigated, we could see the band of μ 2 adaptin if the Western blot film was exposed for an extended period. However, the resulting inferred stoichiometry for μ 2 adaptin was extremely low in comparison to that observed for β 2 adaptin, leading us to conclude that PIP5K γ 661 functionally interacts with AP-2 complex via β 2 adaptin rather than μ 2 adaptin. Similar to the finding that PI(4,5)P₂ binds AP-2 complex via both α and μ 2 adaptins, PIP5K γ 661 may interact with both β 2 and μ 2 adaptins, although preferring one over the other.

It has been reported that talin, which plays a crucial role in focal adhesion formation, also binds to the C-terminus of PIP5K γ 661 and activates the enzyme (Di Paolo *et al*, 2002; Ling *et al*, 2002) via regulation of the PIP5K γ 661 phosphorylation state (Lee *et al*, 2005). However, the mode of interaction of PIP5K γ 661 with AP-2 appears to be subtly different from that with talin, as the dephosphomimetic mutant S645A does not interact with talin (Lee *et al*, 2005), whereas it does with AP-2 (Figure 4E). The different nature of the interaction is also supported by the observation that the interaction of talin is mediated through its FERM domain (Di Paolo *et al*, 2002; Ling *et al*, 2002), but β 2 adaptin does not have a FERM domain. It would be of interest to investigate how the choice of interacting with talin versus AP-2 is determined at the presynapse.

During this study, it was reported that the interaction of talin with PIP5K γ 661 plays a role in SV endocytosis, especially in the early steps of clathrin-coated vesicle maturation (Morgan *et al*, 2004). In the present study, we provide evidence that suggests that interaction of PIP5K γ 661 with AP-2 is required for SV endocytosis (Figure 6). In this experiment, we employed the PIP5K γ 661 C-terminal region and its phosphomimetic and dephosphomimetic mutants, C-S645E and C-S645A, respectively. Because talin as well as AP-2 specifically binds to the wild-type PIP5K γ 661 C-terminal tail, it is possible that the inhibition of depolarization-dependent SV endocytosis by the wild-type PIP5K γ 661 C-terminal tail could be attributed to inhibition of the endogenous enzyme interaction with talin. However, since we observed the same outcome with the dephosphomimetic mutant C-S645A, which does not bind talin (Lee *et al*, 2005) but does bind AP-2 (Figure 4E and F), this indicates that the interaction of AP-2 with PIP5K γ 661 is a critical step in SV endocytosis. Given comparable competitive binding of talin and AP-2 to the PIP5K γ 661 C-terminal region *in vitro* (data not shown), and the possible involvement of both proteins in SV endocytosis, it is plausible that the interaction of each protein with PIP5K γ 661 occurs during a distinct step of SV endocytosis. If this is true, switching of the interaction of talin and AP-2 with PIP5K γ 661 might take place during the process of SV endocytosis via a mechanism that remains to be suggested.

In addition to AP-2 and talin, the small GTPase ARF6 is well known as a PIP5K γ 661-binding partner that directly

activates the lipid kinase activity (Honda *et al*, 1999). It has recently been reported that activation of PIP5K γ , possibly PIP5K γ 661, by ARF6 stimulates clathrin/AP-2 recruitment to the presynaptic plasma membrane (Krauss *et al*, 2003), suggesting that an ARF6–PIP5K γ 661 interaction also plays a crucial role in clathrin-dependent SV endocytosis. If ARF6 also bound to the PIP5K γ 661 C-terminal tail, then it would not be possible to discriminate whether the inhibition of SV endocytosis that we elicited by PIP5K γ 661C-S645A expression in Figure 6 resulted from perturbing the interaction with AP-2 or ARF6. However, the binding site of PIP5K γ 661 for ARF6 appears to be distinct from that for AP-2: although full length of PIP5K γ 661 binds to ARF6, PIP5K γ 661C and PIP5K γ 635C do not (see Supplementary Figure S3). Similarly, we and others have previously reported that ARF6 stimulates PIP5K α (Honda *et al*, 1999), PIP5K β (unpublished data) and PIP5K γ 661 (Krauss *et al*, 2003), suggesting that ARF6 binds to a region common among the PIP5K isozymes, such as the conserved kinase domain, rather than the C-terminal tail unique to PIP5K γ 661. The existence of multiple binding partners for PIP5K γ 661 and their involvement in SV endocytosis (Krauss *et al*, 2003; Morgan *et al*, 2004) leads us to speculate that these molecules cooperate to activate PIP5K γ 661 at endocytic hot spots of the presynaptic plasma membrane to induce SV endocytosis via production of a specific PI(4,5)P₂ pool.

A final question raised by our findings is how PIP5K γ 661 is localized to the limited periaxonal zones of the presynaptic plasma membrane in order to generate the PI(4,5)P₂ pool that is crucial for the efficient induction of SV endocytosis. It is not likely that AP-2 complex regulates location of PIP5K γ 661 to the presynaptic membrane. This is supported by the results that PIP5K γ 661 already locates to the synaptotagmin I-positive presynaptic membrane in the resting state of hippocampal neurons and does not change its location in response to depolarizing stimulation (Figure 5G); in contrast, β 2 adaptin, which distributes diffusely in axonal shafts in the resting state, shifts to the axonal punctate structures upon stimulation (Figure 5F). Given these results, and the finding that ARF6 can interact with PIP5K γ 661 in the resting states of HEK293T cells (see Supplementary Figure S3), it is conceivable that inactive ARF6 anchors PIP5K γ 661 to the presynaptic membrane in the resting state. When neurons are activated, ARF6 may be activated and partially activate PIP5K γ 661 at the presynaptic membrane, resulting in limited PI(4,5)P₂ production, which then cooperates with the activated ARF6 to translocate AP-2 to the periaxonal zone, resulting in further PIP5K γ 661 activation, which would be compatible with the report by Krauss *et al* (2003). This is not an unreasonable proposal, as ARF6 has been reported to be activated at sites of regulated exocytosis (Vitale *et al*, 2002), and may simply remain activated in these regions of inserted membrane, which are the ones that subsequently have the highest probability of undergoing endocytosis.

Materials and methods

Chemicals and antibodies

PI(4)P was purchased from Sigma, λ -PPase was from Upstate and PPI and YOP were from New England Biolabs. Zenon labeling kit and FM4-64 were from Invitrogen. A rabbit polyclonal antibody specific to mouse PIP5K γ and used for immunoprecipitation was raised against a peptide corresponding to amino acids 622–635. The

mouse monoclonal anti-PIP5K γ antibody used for Western blotting and immunocytochemistry was purchased from BD Biosciences. Anti-PI(4,5)P₂ antibody was a generous gift from Dr Umeda, Kyoto University (Miyazawa *et al*, 1988; Emoto *et al*, 2005). Commercial antibodies: anti-Flag M2 antibody (Sigma); anti-HA antibody (clone 6E2); anti-Myc antibody (clone 9B11); anti-GST antibody (clone 26H1); anti- α adaptin, anti- β adaptin, anti- γ adaptin, anti-clathrin heavy chain, anti-AP-180 and anti-amphiphysin antibodies (BD Biosciences); anti-GFP antibody (Invitrogen); and anti-synaptotagmin I antibody (Chemicon).

Plasmids

To construct Flag- and Myc-tagged mammalian expression vectors for PIP5K α , PIP5K β , PIP5K γ 635 and PIP5K γ 661, cDNAs were amplified from mouse brain total RNA using RT-PCR, subcloned into pcDNA3-Flag and pcDNA3-Myc, and sequenced (Honda *et al*, 1999). cDNAs for chimeric PIP5K β / γ 635C and PIP5K β / γ 661C were constructed by a PCR-based strategy and subcloned into pcDNA3-Flag. pEGFP-C1 (Clontech) was used to generate mammalian expression vectors for GFP-tagged PIP5K γ 661C and PIP5K γ 635C. S645A and S645E mutations were introduced into full-length and C-terminal fragment of PIP5K γ 661 by PCR-based site-directed mutagenesis. cDNAs encoding α A, β 1, β 2, β 4 and σ 2 adaptins (Takatsu *et al*, 1998, 2001), μ 2 adaptin (Ohno *et al*, 1998) and β 3 adaptin (Dell'Angelica *et al*, 1997) were generous gifts from Dr Nakayama (Kyoto University) and Dr Ohno (RIKEN Research Center for Allergy and Immunology, Bonifacio, NIH), respectively. These cDNAs were inserted into pGEX-6P-1, pGEX-5X-1 (GE Healthcare BioSciences) and pFlag-MAC (Sigma) for bacterial expression of GST-fused β 2 and σ 2 adaptin, μ 2 adaptin and Flag-tagged α A adaptin, respectively. β 1–4 adaptins were inserted into pcDNA3-HA, which was constructed by insertion of an HA epitope-encoding linker into pcDNA3, for mammalian expression of HA- β 2 adaptin. Fragments of β 2 adaptin were amplified by PCR and inserted into pGEX-6P-1 for bacterial expression of GST fragments. The mammalian expression vectors for p35 and wild-type and kinase-dead Cdk5 (Hashiguchi *et al*, 2002) and for GFP-PLC δ PH (Fujii *et al*, 1999) were generous gifts from Dr Hisanaga (Tokyo Metropolitan University) and Dr Yagisawa (University of Hyogo), respectively.

Preparation of mouse brain lysate and assay for endocytic components interacting with PIP5K γ in brain

Whole brains dissected from P5 ICR mice were homogenized in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl and 1 mM phenylmethylsulfonyl fluoride, and stored at -80°C until use. The brain homogenate (800 μg protein) was resuspended in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl₂, 10 mM KCl, 1% Triton X-100, 5 mM NaF, 2 mM Na₃VO₄, 4 mM Na₂P₂O₇ and protease inhibitors) and centrifuged.

The brain lysate thus prepared was used for detection of endocytic proteins that interact with endogenous PIP5K γ . The lysate was incubated with protein A Sepharose (GE Healthcare BioSciences) and 10 μg of purified rabbit polyclonal anti-PIP5K γ antibody in 1 ml at 4°C for 2 h. After centrifugation, endocytic proteins immunoprecipitated with PIP5K γ were separated by SDS-PAGE and analyzed by Western blotting with the indicated antibodies.

Assay for interaction of PIP5Ks with AP-2 complex

To investigate the *in vitro* interaction of PIP5Ks with AP-2, GST- or Flag-tagged subunits of AP-2 and GST-tagged β 2 adaptin fragments were expressed in *E. coli* and bound to glutathione-Sepharose (GE Healthcare BioSciences) or the anti-Flag M2 affinity gel (Sigma-Aldrich). These protein beads were incubated at 4°C for 2 h with lysates of HEK293T cells expressing tagged PIP5Ks, their C termini (PIP5KC) or PIP5K β / γ C chimeras. After the beads were washed with lysis buffer, the co-precipitated proteins were detected by Western blotting using tag-specific antibodies.

To establish direct interaction between PIP5K γ 661 and β 2 adaptin, Flag-PIP5K γ 661 expressed in *E. coli* was purified with the anti-Flag M2 affinity gel and extensively washed with 0.5 M NaCl in lysis buffer. After Flag-PIP5K γ 661, thus purified, and GST- β 2 adaptin and -Ear domain purified from *E. coli* as described above were incubated at 4°C for 2 h, their direct interaction was assessed by GST pull-down of GST- β 2 adaptin and -Ear domain and subsequent Western blotting with anti-Flag M2 antibody.

To analyze the effects of dephosphorylation of PIP5K γ 661 on its interaction with β 2 adaptin *in vitro*, Flag-PIP5K γ 661 expressed in HEK293T cells was bound to the anti-Flag M2 affinity gel, extensively washed with the high-salt buffer and treated with the phosphatase PP1 at 37°C for 30 min. After Flag-PIP5K γ 661-bound beads were incubated with the lysate of HEK293T cells over-expressing HA- β 2 adaptin at 4°C for 2 h, HA- β 2 adaptin bound to PIP5K γ 661 was analyzed by Western blotting with anti-HA antibody.

To investigate the effects of PIP5K γ 661 phosphorylation by Cdk5 on its interaction with β 2 adaptin in cells, Flag-PIP5K γ 661 and HA- β 2 adaptin were coexpressed with or without Cdk5 and its activator p35 in HEK293T cells. After Flag-PIP5K γ 661 was immunoprecipitated with anti-Flag M2 affinity gel, co-precipitated HA- β 2 adaptin was detected by Western blotting with anti-HA antibody. To confirm the regulation of the interaction by phosphorylation of PIP5K γ 661 on Ser 645, the GST-Ear domain of β 2 adaptin bound to glutathione-Sepharose was incubated with the lysate expressing Flag-PIP5K γ 661 and its mutants, S645A and S645E, or with the lysates expressing GFP-PIP5K γ 661 C-terminal fragments and its mutants, C-S645A and C-S645E, with or without p35/Cdk5. After pulldown, Flag-PIP5K γ 661, GFP-PIP5K γ 661 C-terminal fragment and their mutants were detected as described above using anti-Flag and -GFP antibodies.

The interaction of endogenous PIP5K γ and β adaptin was examined in primary culture of mouse hippocampal neurons. Hippocampal neurons prepared as described below were stimulated with 45 mM KCl at 37°C for 2 min, and endogenous PIP5K γ was immunoprecipitated with polyclonal anti-PIP5K γ antibody. Proteins in the immunoprecipitate were then Western blotted with anti- β adaptin antibody.

Assay of PIP5K γ 661 activity

PIP5K γ 661 activity was determined as previously reported (Honda *et al*, 1999). Briefly, 0.1 pmol of Flag-PIP5K γ 661 and its mutants purified from HEK293T cells or endogenous GFP-PIP5K γ immunoprecipitated from mouse hippocampal neurons before and after depolarizing stimulation was incubated with described proteins at 37°C for 25 min in 50 μl of 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 10 mM MgCl₂, 0.004% (w/v) NP-40 containing 50 μM PI(4)P and 50 μM [γ -³²P]ATP (0.1 μCi /assay). After being extracted by the method of Bligh and Dyer, lipids were separated by thin-layer chromatography as previously reported (Honda *et al*, 1999). [³²P]PI(4,5)P₂ produced was analyzed by a BAS2500 Bio-imaging analyzer (Fuji Photo Film).

Primary culture of mouse hippocampal neurons and transient expression of PIP5K γ 661 C termini, PLC δ PH and β 2 adaptin

Hippocampi dissected from E17.5 ICR mice were treated with 10 U ml⁻¹ papain and 100 U ml⁻¹ DNase in Dulbecco's modified Eagle's medium at 37°C for 20 min. The dissociated hippocampal neurons were cultured in Neurobasal media (Invitrogen) supplemented with B-27 (Invitrogen), 0.5 mM L-glutamine and penicillin/streptomycin (nakalai tesque).

Hippocampal neurons cultured for 17–21 days *in vitro* (DIV) were stimulated with or without 45 mM KCl and subjected to the assay for PIP5K γ interaction with β adaptin, determination of endogenous PIP5K γ activity and immunocytochemical analysis for localization of endogenous PIP5K γ , β adaptin, synaptotagmin I and PI(4,5)P₂. The cultured neurons were also ectopically expressed with GFP-PLC δ PH for visualizing PI(4,5)P₂ and GFP- β 2 adaptin with sindbis pseudovirions and by calcium phosphate method, respectively. After 10–22 h culture, neurons were subjected to live image analysis for the depolarization-dependent PI(4,5)P₂ production and translocation of β 2 adaptin as described below. In addition, the cultured neurons were also infected with sindbis pseudovirions to ectopically express GFP-PIP5K γ 661C and its mutants. After 16–18-h culture, neurons were subjected to the depolarization-dependent endocytosis assay as described below.

Immunocytochemistry

For immunostaining of PI(4,5)P₂, hippocampal neurons treated as described above were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) on ice for 30 min and permeabilized with 0.05% Triton X-100 in PBS for 15 min. For protein staining, hippocampal neurons were fixed with methanol at -30°C for 5 min. Neurons were blocked with 0.1% BSA in PBS for 1 h and

then stained by sequential incubation with primary and secondary antibodies in PBS. For co-immunostaining of PIP5K γ and β adaptin in hippocampal neurons, antibodies fluorescently labeled with the Zenon labeling kit were used. The immunofluorescently stained neurons were then imaged using a fluorescent microscope Axiovert S100 (Zeiss) equipped with an ORCA-ER CCD camera (Hamamatsu Photonics) and a confocal scanner unit CSU21 (Yokogawa Electric Co.).

Live image analysis

Hippocampal neurons expressing GFP-PLC δ PH or GFP- β 2 adaptin were cultured in a 35-mm dish with a thin bottom (Integrated BioDiagnostics) on the stage of a BZ8000 fluorescent microscope (Keyence) with a PlanFluor ELWD 20xC (NA 0.45) (Nikon), controlling the temperature at 37°C and 5% CO₂ concentration using a Microscope Incubation System INU-KI-FI (TOKAI HIT). Before and after depolarizing stimulation of neurons, time-lapse images were collected at the indicated time intervals.

Assay of SV endocytosis

Mouse (17–21 DIV) hippocampal neurons overexpressing GFP, GFP-PIP5K γ 661C or its mutants, C-S645A and C-S645E, were subjected to the assay for depolarization-dependent SV endocytosis according to the method of Chen *et al* (2003). Briefly, neurons were stained in 10 μ M FM 4-64 and 45 mM KCl for 2 min. After collecting FM 4-64

staining images, neurons were subjected to destaining in 45 mM KCl for 2 min for use in background subtraction. GFP and FM4-64 fluorescence signals were obtained using band-pass filters and the fluorescent images acquired were analyzed with IP lab software (Scanalytics Inc.). The intensity of FM 4-64 fluorescence was quantified by measuring the sum of FM 4-64 fluorescence intensity on subtracted images along with an axonal segment delineated by GFP signal.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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