ARF6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol phosphate kinase type Iγ

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Clathrin-mediated endocytosis of synaptic vesicle (SV)* membranes involves the recruitment of clathrin and AP-2 adaptor complexes to the presynaptic plasma membrane. Phosphoinositides have been implicated in nucleating coat assembly by directly binding to several endocytotic proteins including AP-2 and AP180. Here, we show that the stimulatory effect of ATP and GTPγS on clathrin coat recruitment is mediated at least in part by increased levels of PIP2. We also provide evidence for a role of ADP-ribosylation factor 6 (ARF6) via direct stimulation of a synthetically enriched phosphatidylinositol 4-phosphate 5-kinase type Iγ (PIPKIγ), in this effect. These data suggest a model according to which activation of PIPKγ by ARF6-GTP facilitates clathrin-coated pit assembly at the synapse.

Introduction

Synaptic vesicle (SV)* membranes are formed and recycled by clathrin-mediated endocytosis at the presynaptic plasmalemma. This process involves the regulated and directed assembly of clathrin, AP180, and AP-2 adaptor complexes along with several accessory proteins at endocytotic ”hot spots” surrounding the active zone (for review see Hannah et al., 1999; Brodin et al., 2000; Slepnév and De Camilli, 2000; Takei and Haucke, 2001). How precisely coat nucleation is regulated in time and space is unclear, but morphological data suggest that it is a compensatory event tightly coupled to vesicle exocytosis (Heuser and Reese, 1973; Gad et al., 1998).

Growing evidence implicates membrane lipids, in particular phosphoinositides, in the regulation of clathrin-mediated endocytosis (Jost et al., 1998; Cremona and De Camilli, 2001). Several endocytotic proteins such as the α and μ2 subunits of heterotetrameric AP-2 complexes (Gaidarov and Keen, 1999; Collins et al., 2002; Rohde et al., 2002), the ENTH domains of AP180 and epsin (Ford et al., 2001; Ichoh et al., 2001; Mao et al., 2001), and the pleckstrin homology (PH) domain of the large GTPase dynamin (Barylko et al., 1998) can interact directly with phosphoinositides, in particular phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2). Phosphatidylinositol 4-phosphate 5-kinase type Iγ (PIPKIγ) and synaptojanin 1, the major brain PI(4)phosphate 5-kinase and PI(4,5)P2 polyphosphoinositide phosphatase, respectively, are concentrated at synapses, where they undergo activity-dependent dephosphorylation (McPherson et al., 1996; Ishihara et al., 1998; Wenk et al., 2001). Moreover, elevated PIP(4,5)P2 levels induced by genetic inactivation of synaptojanin 1 result in the accumulation of clathrin-coated vesicles at the synapse (Cremona et al., 1999; Harris et al., 2000). These data suggest that clathrin-dependent retrieval of SV membranes may at least in part depend on phosphoinositide metabolism. Given the rapid turnover of phosphoinositides, it seems likely that PI(4,5)P2 synthesis is under tight regulatory control (Cremona and De Camilli, 2001), thereby linking it to the exo-endocytotic cycling of SV membranes.

The formation of clathrin-coated endocytotic intermediates has been reconstituted from lysed nerve terminal membranes incubated with brain cytosol and nucleotides (Takei et al., 1996). Detailed morphometric analysis by EM revealed...
that the presence of clathrin/AP-2–coated buds on native synaptic membranes was potently stimulated by ATP and GTP\(\gamma\)S, a nonhydrolyzable analogue of GTP (Takei et al., 1996). This effect may be contributed to some extent by an inhibition of clathrin-coated vesicle fission induced by the GTP\(\gamma\)S block of dynamin function (Takei et al., 1996). However, by analogy to other vesicular transport events, one might also speculate that GTP\(\gamma\)S could act in part by locking a small GTPase in the GTP-bound conformation (Springer et al., 1999). More specifically, ADP-ribosylation factor (ARF) family members have been implicated in clathrin-coated budding events at the Golgi complex and at the cell periphery (Starnes and Rothman, 1993; Traub et al., 1993; West et al., 1997). ARF has been shown to trigger assembly of vesicle coats onto membranes by directly interacting with coat proteins (Donaldson et al., 1992; Starnes and Rothman, 1993; Traub et al., 1993; Zhao et al., 1997; Austin et al., 2002) or by stimulating phospholipase D (PLD) activity (Kristakas et al., 1996; West et al., 1997; Arneson et al., 1999). Additionally, ARF family members have been found to recruit and activate PI kinases, which mediate PI(4,5)P\(_2\) synthesis (Godi et al., 1999; Honda et al., 1999). However, so far, the physiological role of ARF proteins in the recruitment of clathrin/AP-2 coats at the plasma membrane (Robinson and Kreis, 1992; West et al., 1997) and in SV recycling has remained unclear. Among the different ARF family members known to date, only ARF6 is localized to the plasma membrane, where it has been implicated in regulating actin dynamics and membrane turnover (D’Souza-Schorey et al., 1998; Randazzo et al., 2000; Brown et al., 2001). ARF6 has also been demonstrated to regulate clathrin-mediated endocytosis from the apical (Altschuler et al., 1999) and basolateral surface (Palacios et al., 2002) of polarized MDCK cells. Consistent with a putative role of ARF6 in SV recycling, mSec7, a brefeldin A–insensitive ARF6-specific guanine nucleotide exchange factor (GEF) has been demonstrated to function at the synapse (Ashery et al., 1999). Here, we show that activated ARF6 facilitates clathrin/AP-2–coated pit nucleation from synaptic membranes via the stimulation of PIP\(_2\) production mediated by PIPKI\(\gamma\) activation.

**Results**

**Clathrin/AP-2 recruitment to synaptic membranes is stimulated by ATP and GTP\(\gamma\)S**

Assembly of clathrin- and AP-2–coated endocytotic intermediates was previously reconstructed from lysed nerve terminal lysed synaptosomal membrane fraction (LP2) membranes incubated with brain cytosol and nucleotides (Takei et al., 1996). LP2 membranes mainly consist of synaptic vesicles (SV) and endosomal membranes. Carbonate treatment of LP2 efficiently removed associated clathrin, AP180, and AP-2 (Fig. 1 A), enabling us to biochemically analyze membrane recruitment of clathrin/AP-2 coat proteins from added cytosol in vitro (see also Hauke and De Camilli, 1999). To study the nucleotide dependence of clathrin coat recruitment, LP2 membranes were incubated with brain cytosol and re-isolated by centrifugation. Membrane-associated proteins were detected by immunoblotting. A pool of AP-2 and clathrin was found associated with LP2 membranes when the reaction was performed in the absence of nucleotides (Fig. 1 B). The concomitant presence of 2 mM ATP and 200 \(\mu\)M GTP\(\gamma\)S greatly stimulated recruitment of both coat components onto the membrane (Fig. 1, A–C). GTP\(\gamma\)S alone was not sufficient to enhance AP-2 and clathrin binding, whereas ATP alone or ATP plus GTP resulted in a small increase of membrane-associated coat proteins. (Fig. 1, B and C). By contrast, membrane binding of the cytosolic chaperone Hsc70 (analyzed as a control protein) only required ATP, but was not affected by guanine nucleotides (Fig. 1 B). The presence of ATP and GTP\(\gamma\)S also stimulated recruitment of the monomeric clathrin adaptor AP180 (and PIPKI\(\gamma\); see below for details), whereas the association of tubulin or endophilin with the membrane was not affected by these nucleotides (Fig. 1 D). Together, these results confirm earlier morphometric data on the nucleotide-dependent formation of clathrin-coated buds (Takei et al., 1996), and suggest that activation of a GTPase is involved in the nucleation of clathrin/AP-2 coats at the presynaptic plasmalemma.

Because GTP\(\gamma\)S alone was not sufficient to stimulate coat recruitment, we hypothesized that the addition of ATP was needed to drive phosphorylation reactions. Indeed, the presence of A3, a broad spectrum kinase inhibitor, potently inhibited ATP/GTP\(\gamma\)S-stimulated recruitment of AP-2 and clathrin (Fig. 1 E).

**Clathrin/AP-2 recruitment to synaptic membranes is not inhibited by brefeldin A**

ARF family members have been shown to facilitate vesicle budding by stimulating recruitment of coat components to the membrane (Springer et al., 1999). Therefore, we tested the effect of brefeldin A (BFA) on clathrin/AP-2 recruitment in our assay. BFA inhibits nucleotide exchange factors acting on ARF family members except most of those specific for ARF6 (Randazzo et al., 2000). Consistent with earlier observations on AP-2 association with the plasma membrane of intact cells (Robinson and Kreis, 1992), no effect on the ATP- and GTP\(\gamma\)S-dependent recruitment of AP-2 and AP180 to LP2 membranes (Fig. 2) was seen. When isolated Golgi membranes were used instead of synaptic membranes, addition of BFA completely blocked clathrin binding to the Golgi complex (unpublished data; Robinson and Kreis, 1992). In agreement with these results, it has been found that BFA does not affect SV recycling, the main trafficking pathway mediated by clathrin/AP-2 coats at the synapse (Mundigl et al., 1993).

Together, our data implicate a GTPase distinct from ARF1-5 (Randazzo et al., 2000) in the stimulation of ATP/GTP\(\gamma\)S-induced coat recruitment to synaptic membranes. Such a GTPase could be ARF6, the BFA-insensitive ARF family member known to act at the cell surface. Consistent with this possibility, we observed recruitment of a substantial amount of ARF6 along with clathrin, AP180, and AP-2 to LP2 membranes on incubation with cytosol in the presence of ATP and GTP\(\gamma\)S (Fig. 1 A).

**ARF6 is enriched in synaptic plasma membrane subfractions**

A role of ARF6 in presynaptic clathrin-mediated vesicle recycling is plausible given the enrichment of the ARF6-spe-
cific nucleotide exchange factor mSec7 in nerve terminals (Ashery et al., 1999). pAbs that recognize ARF6 but not ARF1 (Fig. 3 A) detect high level expression of ARF6 in several tissues, including brain, as previously reported (Cavagnagh et al., 1996; Yang et al., 1998; and unpublished data). The subcellular distribution of ARF6 as well as that of other well-characterized synaptic markers was analyzed in fractions of pig brain (obtained by the procedure of Maycox et al. [1992]). ARF6 was found in synaptosomes (P2) and within synaptosomes was primarily localized to the plasma membrane–containing fraction (LP1; Fig. 3 B). The LP2 fraction, mostly comprising SV and endosomal membranes, was highly enriched in the SV protein synaptophysin, but contained only relatively low levels of ARF6. ARF6 was also present (but not enriched) in highly purified clathrin-coated vesicles isolated from nerve terminals (Fig. 3 B) or whole brain (not depicted). Consistent with these biochemical data, activated HA-tagged ARF6(Q67L) accumulated at synapses in transfected cortical neurons grown in vitro, as demonstrated by its enrichment in synaptophysin-positive structures (Fig. 3 C). We conclude that ARF6 is present at synapses, where it is found predominantly in plasma membrane fractions from which SV recycling occurs.

Effect of ARF6 mutants on clathrin/AP-2 recruitment to synaptic membranes

To investigate directly whether ARF6 can trigger AP-2/clathrin coat formation, we tested recombinant myristoylated ARF6 mutants for their ability to facilitate clathrin/AP-2 recruitment to synaptic membranes (Fig. 1 A). Reactions were performed in the presence of 200 μM GTP instead of GTPγS to prevent generalized activation of all endogenous GDPases. Addition of 1 μM recombinant ARF6(Q67L), an ARF6 mutant locked in the GDP-bound state due to defective GTP hydrolysis, was sufficient to promote recruitment of clathrin, AP-2, and AP180 as efficiently as addition of GTPγS in the absence of exogenous ARF
By contrast, ARF6(T27N), a GTP-binding defective dominant-negative mutant, failed to increase binding of coat proteins to membranes. ARF6(Q67L)-stimulated coat recruitment was dose-dependent with half-maximal stimulation seen at $120 \text{ nM ARF6}$ (Fig. 4 B). High concentrations of ARF6(T27N) (above $2 \text{ M}$) instead inhibited the ATP/GTP-induced association of clathrin and AP-2 with the membrane in a concentration-dependent manner (Fig. 4 C). Like corresponding mutants of other GTPases, ARF6(T27N) may trap GEFs in a stable complex, preventing activation of endogenous ARF6.

**ARF6-GTP interacts directly with PIPKιγ**

To obtain possible insights into the mechanism by which ARF6-GTP stimulates clathrin/AP-2 recruitment to synaptic plasma membranes, we used an affinity purification approach. In pull-down experiments, NH2-terminal GST fusion constructs of the constitutively active ARF6(Q67L) or the GTP-binding defective mutant ARF6(T27N) were analyzed for their ability to retain various endocytotic proteins. Considering that ARF1 directly interacts with AP-1 complexes at the trans-Golgi network and AP-3 complexes on endosomal membranes, we initially focused on coat components as putative interactors of ARF6. No association of either ARF6 mutant with clathrin or subunits of AP-2 could be detected. Likewise, we did not detect any interaction of GST-ARF6 with AP180, amphiphysin I, dynamin I, and auxilin, or the SV protein synaptotagmin I (Fig. 5 A).

A variety of endocytotic proteins including AP-2 have been found to interact with the membrane via phosphoinositides (Gaidarov and Keen, 1999; Ford et al., 2001; Itoh et al., 2001; Mao et al., 2001; Collins et al., 2002; Rohde et al., 2002). Moreover, members of the ARF family have been shown to stimulate phosphatidylinositol kinases in different systems (Godi et al., 1999; Honda et al., 1999; Skippen et al., 2002). Therefore, we investigated whether ARF6 interacts with PIPKιγ, an isoform of PI(4)P 5-kinase highly enriched in brain (Ishihara et al., 1998) and concentrated in synaptic plasma membrane fractions. (A) Polyclonal anti-ARF6 antibodies specifically recognize ARF6, but not ARF1. Top, Ponceau-stained nitrocellulose membrane. 3 μg ARF protein was loaded per lane. Bottom, immunoblot analysis using anti-ARF6 antibodies. (B) Subcellular fractionation of pig brain homogenate according to Maycox et al. (1992). 15 μg protein was loaded per lane and analyzed by immunoblotting against clathrin heavy chain (HC), μ2-adaptin, dynamin I, synaptophysin, and ARF6. H, brain homogenate; P2, crude synaptosomes; P2', washed synaptosomes; S3, cytosol; LP1, 20,000-g pellet after lysis of synaptosomes; LP2, 55,000-g pellet; CCV, purified clathrin-coated vesicles. (C) Localization of ARF6(Q67L) in transfected cortical neurons. Neurons at 11 days in vitro were fixed and analyzed for the distribution of HA-tagged ARF6(Q67L) and the presynaptic marker protein synaptophysin using a Leica confocal laser microscope. Bar, 10 μm. Note that synaptophysin-positive synapses devoid of ARF6(Q67L) may originate from nontransfected neurons.
the presynaptic compartment (Wenk et al., 2001). Indeed, GST-ARF6(Q67L) efficiently pulled down PIPKIγ (Fig. 5 A). By contrast, little interaction was seen between PIPKIγ and the inactive GDP-bound mutant GST-ARF6(T27N). Similar results were seen if N-myristoylated hexahistidine-tagged ARF6 mutants were used instead (unpublished data).

The interaction between ARF6 and PIPKIγ appeared to be direct because GST-ARF6 bound to radiolabeled PIPKIγ synthesized by coupled transcription/translation in vitro (unpublished data). To determine the isoform specificity for different ARF family members, hexahistidine-tagged myristoylated ARF6(Q67L) or ARF1(Q71L) were compared for their ability to bind to PIPKIγ or the trans-Golgi clathrin adaptor AP-1, a protein complex known to bind to both ARF1 and ARF6 (Austin et al., 2002). Although both ARF1(Q71L) and ARF6(Q67L) displayed similar affinities for AP-1, as detected by Western blotting of the affinity-purified material for the AP-1 subunit γ-adaptin, only ARF6 was able to effectively pull-down PIPKIγ (Fig. 5 B). By contrast, a control protein (arfaptin2) did not

Figure 4. ARF6-GTP stimulates clathrin/AP-2 recruitment to synaptic membranes. (A) Coat recruitment to LP2 membranes was performed and analyzed as described in the legend to Fig. 1. Samples containing 1 μM ARF6 mutants were incubated in the presence of 200 μM GTP and analyzed by quantitative immunoblotting against clathrin heavy chain (HC), AP180, α-adaptin, Hsc70, and synaptotagmin I. (B) Dose dependence of the stimulatory effect of ARF6(Q67L) on clathrin recruitment to membranes as shown in A. Values were normalized to the amount of clathrin bound in the presence of ATP and GTPγS (100%). (C) Dose dependence of the inhibitory effect of ARF6(T27N) on clathrin recruitment to membranes as shown in A. Values were normalized to the amount of clathrin bound in the presence of ATP and GTPγS (100%).

Figure 5. Activated ARF6 interacts with PIPKIγ in brain. (A) ARF6(Q67L) but not ARF6(T27N) specifically affinity purifies PIPKIγ. Western blot analysis of proteins pulled down by GST and GST–ARF6 fusion proteins (80 μg) from a detergent extract of rat brain. Samples were analyzed by SDS-PAGE and immunoblotting. 5% Std., 5% of the extract used for affinity purification. (B) PIPKIγ specifically interacts with ARF6(Q67L). Immunoblot analysis of PIPKIγ affinity purified with myristoylated His6-tagged ARF6(Q67L), ARF1(Q71L), or arfaptin 2 as described under A. 5% Std., 5% of the extract used for affinity purification. (C) PIPKIγ can be cross-linked to ARF6(Q67L) during recruitment of clathrin/AP-2 to synaptic membranes. LP2-membranes were incubated with brain cytosol, myristoylated His6-tagged ARF6, and nucleotides. DTSP was added where indicated. His6-tagged ARF6 was recovered and cross-linked proteins were analyzed by immunoblotting. Top, immunoblot analysis with antisera against PIPKIγ, AP180, large and medium subunits of the AP-2 complex β2-adaptin, α-adaptin, and μ2-adaptin, respectively, and clathrin heavy chain (HC). Bottom, Coomassie-stained gel demonstrating that equal amounts of ARF6 have been recovered in each sample.
interact with either AP-1 or PIPKIγ. Together, these results show that ARF6 specifically binds to PIPKIγ in vitro in a GTP-dependent manner, but not to clathrin/AP-2 coat components.

To finally determine whether ARF6 associated with PIPKIγ during clathrin/AP-2 recruitment to synaptic membranes, we performed chemical cross-linking experiments. To this aim, LP2 membranes were first incubated with cytosol, ATP, ARF6(T27N)-His6 or ARF6(Q67L)-His6 and GDP or GTP, respectively. 3,3′-Dithio-bis(propionic acid N-hydroxysuccinimide ester) (DTSP), a cleavable amine-reactive cross-linking reagent was added, samples were solubilized under denaturing conditions, and ARF6-His6 was recovered by Ni-NTA affinity chromatography. ARF6(Q67L) but not its inactive GDP-bound counterpart (T27N) became efficiently cross-linked to PIPKIγ (Fig. 5 C). No interaction was seen with clathrin, AP180, or any of the individual subunits of the AP-2 adaptor complex. Our combined data suggest that ARF6-GTP directly interacts with PIPKIγ on synaptic membranes.

ARF6-GTP and PIPKIγ colocalize in transfected cells
The biochemical interaction of ARF6 with PIPKIγ was supported by morphological studies of cotransfected cells. Consistent with previous data (Brown et al., 2001), ARF6(Q67L)-EGFP expressed in Cos7 cells was found in peripheral plasma membrane invaginations, vacuolar structures and membrane ruffles. In these structures, it colocalized with cotransfected PIPKIγ-p90 (Fig. 6 A). By contrast, little if any colocalization was seen between PIPKIγ and the inactive GDP mutant of ARF6 (Fig. 6 B). As expected, PIPKIγ-containing structures were highly enriched in PI(4,5)P₂, as visualized by coexpression of PHPLCδ1-EGFP, a specific interactor for this phosphoinositide (Fig. 6 C).

ARF6 directly activates PIPKIγ on liposomes
Given the interaction of ARF6-GTP with PIPKIγ, we asked whether ARF6 might regulate phosphatidylinositol 4-phosphate 5-kinase activity in bovine brain cytosol. To this end, cytosol was incubated with γ[32P]ATP, phosphatidylinositol

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**Figure 6.** ARF6(Q67L) colocalizes with PIPKIγ in transfected cells. Cos7 cells were cotransfected with plasmids encoding ARF6(Q67L)-EGFP (A), ARF6(T27N)-EGFP (B), or PHPLCδ1-EGFP (C) and HA-tagged PIPKIγ. 24 h after transfection, the cells were fixed and analyzed by immunofluorescence microscopy. Merged images are shown in A–C (middle panels). Bar, 20 μm.
4-phosphate (PI4P)–containing liposomes, and neomycin to prevent PIP2 degradation. Lipid products were analyzed by TLC, and the amount of incorporated radioactivity was quantified. Addition of 1 μM recombinant myristoylated ARF6(Q67L)-GTP greatly stimulated PI4P 5-kinase activity compared with ARF6(T27N). A slightly less pronounced increase in PI4P kinase activity was seen if wild-type ARF6-GTP was used instead (Fig. 7 A). Under these conditions (i.e., addition of neomycin), no formation of phosphatidic acid from 3H-labeled phosphatidylcholine was detectable (unpublished data).

To analyze whether ARF6 directly activates PIPKγ, we monitored the activity of recombinant PIPKγ-p90 in an in vitro assay using liposomes made of total brain lipids as a substrate. Liposomes were incubated with recombinant PIPKγ and myristoylated ARF6, or mutants thereof, in the presence of 200 μM GTP and γ[32P]ATP. Lipid products were analyzed as described above. Myristoylated ARF6(Q67L) induced a strong increase in kinase activity compared with the basal activity seen in the absence of ARF (Fig. 7 B). A similar (albeit weaker) effect was produced by wild-type ARF6. By contrast, the GTP-binding defective mutant ARF6(T27N) failed to enhance kinase activity (Fig. 7 B). The stimulatory effect of ARF6 on PIP2 generation from total brain lipids was smaller than that observed with liposomes containing high concentrations of PI(4)P (compare Fig. 7 A with Fig. 7 B), presumably due to incomplete substrate saturation of the enzyme. These data suggest that ARF6-GTP stimulates PIPKγ activity under conditions mimicking physiological substrate levels.

Although addition of activated ARF6 increased formation of PIP2 from total brain lipids, no effect on PIP generation was detectable (Fig. 7 C). Moreover, immunodepletion of PIPKγ from cytosol almost completely abolished PIP2 formation (see also Wenk et al., 2001), and this defect could not be restored by addition of exogenous ARF6-GTP or mutants thereof (Fig. 7 C). This indicates that ARF6-mediated stimulation of PIP2 synthesis is indeed primarily due to activation of PIPKγ. In summary, these results demonstrate a direct interaction between activated ARF6 and PIPKγ in brain cytosol that results in a strong stimulation of PI(4)P 5 kinase activity and PIP2 formation.
ARF6-GTP stimulates PIP$_2$ formation on synaptic membranes

Also, we investigated whether ARF6-mediated clathrin/AP-2 coat recruitment (Fig. 1 and Fig. 4) is accompanied by an increase of PIP$_2$ levels in synaptic membranes. We stimulated coat recruitment to LP2 membranes with $\gamma^{[32P]}$ATP and GTP in the absence or presence of the different ARF6 mutants. As shown in Fig. 7 D, 1 μM recombinant ARF6(Q67L) stimulated PIP$_2$ synthesis up to 2.5-fold compared with the amount of PIP$_2$ formed in the presence of cytosol alone. In contrast, ARF6(T27N) failed to affect PIP$_2$ levels in LP2 membranes. Addition of wild-type ARF6-GTP resulted in an intermediate stimulation of PIP$_2$ formation (Fig. 7 D). Collectively, our data suggest that ARF6-GTP stimulates PIP$_2$ synthesis at the synapse by activating PIPK I$\gamma$.

Masking or degradation of PIP$_2$ interferes with clathrin/AP-2–coated pit assembly and receptor-mediated endocytosis

Finally, if the ARF6-mediated stimulation of PIPK I$\gamma$ played a major role in mediating the effect of ATP/GTP$_S$ on clathrin/AP-2 recruitment, one would expect that masking PIP$_2$ with a PIP$_2$-binding module or degradation of PIP$_2$ by an inositol phosphatase would inhibit such recruitment to synaptic membranes or in living cells. We performed recruitment

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**Figure 8. Effects of PIP$_2$ masking or degradation on clathrin/AP-2–coated pit assembly.** (A) Recombinant human PHPLC$_1$ inhibits ATP/GTP$_S$-induced membrane recruitment of clathrin/AP-2 coat components. Clathrin/AP-2 recruitment onto presynaptic membranes was performed as described in Fig. 1 in the presence or absence of ATP/GTP$_S$, purified PHPLC$_1$, or BSA. Samples were analyzed by quantitative Western blotting using antisera against clathrin heavy chain (HC), $\alpha$-adaptin, synaptotagmin I as a membrane marker, and Hsc70 as a control. (B and C) Overexpression of membrane-targeted HA-tagged inositol 5-phosphate phosphatase domain of synaptojanin 1 (HA-IPP1-CAAX) mislocalizes clathrin and AP-2. Cos7 cells expressing HA-IPP1-CAAX were fixed 24 h after transfection and analyzed for the distribution of AP-2 (B) or clathrin (C) by immunofluorescence microscopy. Bar, 20 μm.
experiments in the presence of the recombinant PH domain of human PLC61 (PHPLC61), a specific PI(4,5)P2-binding protein. Addition of recombinant PHPLC61 to synaptic membranes inhibited the ATP/GTP-S-induced binding of clathrin and AP-2 in a concentration-dependent manner, whereas addition of GST had no effect (Fig. 8 A). Likewise, overexpression of the prenylated HA-tagged inositol 5-phosphatase domain of synaptojanin 1 (HA-IPP1-CAAX), a PI(4,5)P2-degrading enzyme, in Cos7 cells resulted in the mislocalization of AP-2 to patch-like structures (Fig. 8 B). Similar (albeit less dramatic) effects were seen on peripheral clathrin-positive puncta. By contrast, a pool of clathrin remained associated with the trans-Golgi network in transfected cells (Fig. 8 C).

Consistent with its effects on clathrin/AP-2–coated pit formation and with previous observations (Malecz et al., 2000), cells expressing HA-IPP1-CAAX displayed a strongly reduced ability to internalize Texas red epidermal growth factor (EGF; Fig. 9 A) or Alexa® 488-transferrin (Fig. 9 B). Similar (albeit slightly less pronounced) effects on transferrin uptake were seen on overexpression of EGFP-tagged PHPLC61 (unpublished data).

Discussion
In the present work, we have investigated mechanisms involved in the nucleotide-dependent regulation of clathrin-coated pit nucleation at the synapse. Our results implicate ARF6 in this process and demonstrate two effects of the GTP-bound form of this small GTPase; stimulation of the recruitment of clathrin/AP-2 to presynaptic membranes and binding plus activation of PIPKIγ. They also suggest that the two effects are related and that PI(4,5)P2 production by PIPKIγ stimulation represents the major mechanism through which ARF6 enhances clathrin/AP-2 recruitment. The action of GTP-ARF6 on clathrin/AP-2 recruitment mimics the effect of GTPγS, and its effects are antagonized by experimental manipulations that prevent either ARF activation (i.e., dominant-negative ARF6) or PI(4,5)P2 production and availability (i.e., kinase inhibition, PIPKIγ depletion, and PI(4,5)P2 hydrolysis by synaptojanin’s inositol 5’-phosphatase domain). These results strongly indicate that enhanced clathrin coat recruitment mediated by a stimulation of PI(4,5)P2 synthesis plays a major role in the GTPγS-stimulated nucleation of morphologically detectable clathrin-coated pits on synaptic membranes (Takei et al., 1996), although a block or delay in fission may also contribute to this ultrastructural change.

The strong stimulatory effect of ARF6 on PIPKIγ extends to this brain-enriched enzyme a property previously demonstrated for predominantly nonneuronal isoforms of PIPK (Honda et al., 1999; Skippen et al., 2002). It was also shown that PLD stimulation by ARF family proteins, including ARF6, may play a role in enhanced PI(4,5)P2 production by generating phosphatidic acid, a reported activator of PI(4)P 5-kinases (Brown et al., 1993; West et al., 1997; Arneson et al., 1999). This effect may synergize with the direct effect of ARF6 on PIPKIγ in the generation of PI(4,5)P2 at the synapse. Although ARF6 appears to have a broad distribution in the nervous system, the weak immunocytochemical signal produced by available antibodies to endogenous ARF6 in
localization" of endocytotic clathrin coats was shown to be
PIPKIα-mediated endocytosis from the plasma membrane.

The precise interplay between mSec7 and other brain
ARF6-specific GEFs, and their role in the activation of
ARF6 at the presynaptic plasma membrane remains to be
investigated. It seems possible that ARF6 may synergize with
the synthetically enriched foci al adhesion protein talin in acti-
vating PIPKIγ at the synapse (Di Paolo et al., 2002; Ling et
al., 2002). The finding that enhanced PI(4,5)P2 production
may play a critical role in the recruitment of endocytotic
clathrin coats is supported by a large body of data besides
those presented here. Biochemical and structural studies
have demonstrated an interaction of the clathrin adaptor
AP-2, AP180, and dynamin with PI(4,5)P2 (Barylko et al.,
1998; Gaidarov and Keen, 1999; Ford et al., 2001; Mao et
al., 2001; Rohde et al., 2002). Genetic disruption of the
polyphosphoinositide phosphatase synaptojanin (Cremona
et al., 1999; Harris et al., 2000) and other manipulations
that disrupt its function and therefore lead to an accumula-
tion of PI(4,5)P2 in neurons, enhance the presence of clath-
rin coats on synaptic membranes both in vivo (Cremona et
al., 1999; Gad et al., 2000) and in cell-free systems (Cre-
mona et al., 1999; Wenk et al., 2001; Kim et al., 2002). Our
data are also consistent with (and further support) recent re-
ports suggesting that ARF6 (Altschuler et al., 1999; Palacios
et al., 2002) and PIPK (Barbieri et al., 2001) regulate clath-
rin-mediated endocytosis from the plasma membrane.

As cell fractionation data indicate, neither ARF6 nor
PIPKIγ are enriched in crude SV (Wenk et al., 2001, and this
paper). Instead, ARF6 is localized in plasma membrane frac-
tions (Cavenagh et al., 1996; Yang et al., 1998), consistent
with a role in priming the membrane for coating. PIPKIγ is
found primarily in the cytosol (Wenk et al., 2001), but as
shown by EM (Wenk et al., 2001) and biochemically (this
paper), is recruited by GTPγS, and therefore most likely by
ARF6-GTP, to the membranes at which clathrin-coated pits
nucleate. A GTPγS-stimulated recruitment of clathrin/AP-2
coats to endosomes was demonstrated by Robinson and co-
workers in nonneuronal cells (West et al., 1997). This "mis-
localization" of endocytotic clathrin coats was shown to be
dependent on enhanced PI(4,5)P2 production on endo-
somes, and has been attributed to PLD activation by an ARF
GTPase. Excess PI(4,5)P2 on endosomes can also be gener-
ated by overexpression of an active form of ARF6 (Brown et
al., 2001). As shown by Honda et al. (1999) and by our pre-
sent results, a major mechanism through which active
ARF6 may stimulate PI(4,5)P2 production is via the recruit-
ment and activation of a PI(4)P 5-kinase activity. We specu-
late that in neuronal cells, PI(4,5)P2 is segregated at the plasma
membrane by the coordinate action of PI(4)P 5-kinas,
which are primarily localized at the cell surface, and of the
phosphoinositide phosphatase synaptojanin, which cleaves
PI(4,5)P2 on endocytotic membranes (Kim et al., 2002;
Stefan et al., 2002). Excess active ARF6—either because of
GTPγS addition or because of mutation that inactivates its
GTPase activity—may alter this balance and lead to an ab-
normal accumulation of PI(4,5)P2 on endosomes.

It seems particularly interesting that PIPKIγ is stimulated
by ARF6, a protein previously implicated in regulating actin
dynamics and membrane turnover (D’Souza-Schorey et al.,
1998; Randazzo et al., 2000; Schafer et al., 2000). Invagina-
tions of the plasma membrane that resemble those found at
synapses after prolonged stimulation (Heuser and Reese,
1973; Takei et al., 1996; Gad et al., 2000) have been de-
tected in nonneuronal cells on expression of constitutively
active ARF6 (D’Souza-Schorey et al., 1998) or of an ARF6-
specific exchange factor (Franco et al., 1999). ARF6 may re-
cruit and activate PIPKIγ at the synapse, thus increasing the
local PI(4,5)P2 concentration. This, in turn, would facilitate the
formation of endocytotic structures like clathrin-coated pits
and deeper membrane invaginations from which clathrin-
coated vesicle budding can also occur. A number of observa-
tions suggest that clathrin-mediated endocytosis is highly in-
terconnected to dynamics of the actin cytoskeleton (Lamaze
et al., 1997). Accordingly, many accessory proteins of the
clathrin pathway directly couple endocytotic coat formation
to actin rearrangements (Qualmann et al., 2000; Schafer et
al., 2000; Hussain et al., 2001; Lee and De Camilli, 2002;
Orth et al., 2002). Endocytotic “hot spots” at synapses coin-
dice with actin-rich zones (Kelly, 1999; Gad et al., 2000),
and the accumulation of clathrin-coated vesicles induced in
nerve terminals by the disruption of synaptojanin function
 correlates with the presence of a meshwork of actin around
these vesicles (Cremona et al., 1999; Gad et al., 2000; Kim
et al., 2002; Shupliakov et al., 2002). ARF6 may regulate ac-
tin dynamics through multiple cooperative mechanisms. Via
the increase in PI(4,5)P2 production mediated by stimula-
tion of PLD (Brown et al., 1993) and PIPKs (Honda et al.,
1999; this paper), it enhances the recruitment and activation of
actin regulatory proteins including N-WASP and small
GTPases of the Rho family (Takeawa and Itoh, 2001). It
can also directly regulate Rac via its binding to arfaptin
2/Por1 (Shin and Exton, 2001). Possibly through its effects
on Rac and actin, ARF6 can stimulate formation of macro-
pinosomes (Brown et al., 2001).

Thus, ARF6 appears to have a major regulatory role in en-
docytosis because it can control both clathrin-dependent
and -independent endocytotic pathways. It will be critical to
further elucidate the localization and regulation of ARF6-
GEFs and the effect of synaptic activity on such regulation.

Materials and methods

Materials

DTSP, l-phosphatidylinositol 4-phosphate, and total brain lipid extracts
were purchased from Sigma-Aldrich; γ[32P]ATP was purchased from Amer-
sham Biosciences. pAbs recognizing ARF6 were a gift of Dr. Julie Donald-
sen (National Institutes of Health, Bethesda, MD).

Recombinant proteins

Wild-type or mutant ARF6s or ARF1 GST fusion proteins were purified from
cultures infected with a recombinant expression vector. An antibody
against GST was used to assess expression levels of recombinant
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Recruitment experiments

Rat brain cytosol and LP2 membranes were prepared as described previously (Haucke and De Camilli, 1999). Membranes (1 mg/ml protein) were washed at 4°C in 0.1 M sodium carbonate, pH 9.5, for 15 min, recovered by centrifugation (89,000 g, for 15 min at 4°C), and resuspended in cytosolic buffer. Recruitment experiments were performed in a total volume of 400 µl cytosolic buffer containing 60 µg/ml LP2 membranes and 1.2 mg/ml rat brain cytosol. Nucleotides and recombinant myristoylated ARF6 proteins were included where indicated. The samples were incubated for 15 min at 37°C, loaded on a cushion of 0.5 M sucrose in cytosolic buffer, and centrifuged at 150,000 g (for 1 h at 4°C). Pellets were washed with 500 µl cytosolic buffer and centrifuged at 175,000 g for 15 min. Finally, the pellet was resuspended in sample buffer and proteins recruited to LP2 membranes were analyzed by quantitative Western blots using 123I-protein A for detection and phosphoimage analysis (Image Reader 3000; Fuji).

Pull-down experiments

For GST pull-downs, 0.5 mg/ml GST or GST-ARF6 bound to resin was supplemented with 1 mM GDP (GST-ARF6(T27N)) or 1 mM GTP (GST-ARF6(Q67L)) and incubated with 3 mg/ml mg rat brain extract (in 20 mM Hepes/KOH, pH 7.4, 320 mM sucrose, 2 mM MgCl2, 1% Triton X-100, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 100 µg/ml Pefabloc®) for 4 h at 4°C. The beads were extensively washed and finally extracted twice with 100 µl Laemmli sample buffer. Alternatively, 0.15 mg/ml Histagged Arfaptin 2 or myristoylated ARF6(Q67L)-His6, or ARF1(Q71L)-His6, bound to Ni²⁺-NTA beads were rotated end over end with 2.5 mg/ml rat brain extract supplemented with 15% glycerol for 1 h at 4°C. The resin was washed and extracted twice with 120 µl sample buffer.

Cross-linking experiments

1-µg LP2 membranes were incubated with 5 mg rat brain cytosol in the absence or presence of 200 µM myristoylated ARF6(T27N) or ARF6(Q67L)-His6, 2 mM ATP, and 200 µM GDP or GTP in a final volume of 1.5 ml for 15 min at 37°C. Proteins were cross-linked with 0.5 mM DTSP for 1 h at 4°C. Samples were solubilized with 1% Triton X-100, centrifuged at 20,000 g for 15 min at 4°C, and denatured in 6 M guanidinium hydrochloride for 1 h at 20°C. His-tagged ARF6 and cross-linked partners were recovered by extracting twice with Ni²⁺-NTA beads in the presence of 10 mM imidazole. The collected beads were washed thoroughly and extracted with sample buffer.

Phosphoinositide kinase assays

For phosphoinositide kinase assays, liposomes (Fig. 7 D) were used. Large unilamellar liposomes were prepared from cholesterol/phosphatidylincholine/phosphatidylinositol 4-phosphate (20:7:46, wt/wt/wt; Fig. 7 A) or total brain lipids (Fig. 7 B and C). 1.2 mg/ml brain cytosol was pre-incubated for 2 min at 37°C with 200 µM GTP, 100 µM neomycin, 2 mM ATP, and γ[32P]ATP (0.2 µCi/sample) in cytosolic buffer (25 mM Hepes/KOH, pH 7.2, 25 mM KCl, 2.5 mM magnesium acetate, and 150 mM potassium glutamate). 1 mM recombinant myristoylated ARF6 protein was included where indicated. Phosphorylation reactions (15 min at 37°C in a total volume of 500 µl) were started by adding liposomes. Lipid products were extracted and analyzed as described by Ki- nuta et al. (2002). For some experiments, liposomes were substituted with LP2 membranes (480 µg protein, 385 µg total phospholipids) as substrate. The activity of recombinant PIPKγ-p90 or brain PIPKβ was determined in the presence or absence of ARF6 proteins essentially as previously described using total brain lipids as a substrate (Wenks et al., 2001).

Transfection and internalization assay

Cos-7 cells were transiently transfected with plasmids encoding HA-tagged PIPKγ-p90 or brain PIPKβ determined in the presence or absence of ARF6 proteins essentially as previously described using total brain lipids as a substrate (Wenks et al., 2001).

Nucleofection of isolated cortical neurons

Cortical neurons were isolated from newborn rats and transfected using a nucleofection system (Amaxa) according to the manufacturer’s instructions.

References


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