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# A phosphatidylinositol (4,5)-bisphosphate binding site within μ2-adaptin regulates clathrin-mediated endocytosis

**Gundula Rohde,[1] Dirk Wenzel,[2] and Volker Haucke[1]**

[1]Zentrum für Biochemie and Molekulare Zellbiologie, Department of Biochemistry II, University of Göttingen, D-37073 Göttingen, Germany
[2]Department of Neurobiology, Max-Planck-Institute for Biophysical Chemistry, D-37077 Göttingen, Germany



The clathrin adaptor complex AP-2 serves to coordinate clathrin-coated pit assembly with the sorting of transmembrane cargo proteins at the plasmalemma. How precisely AP-2 assembly and cargo protein recognition at sites of endocytosis are regulated has remained unclear, but recent evidence implicates phosphoinositides, in particular phosphatidylinositol (4,5)-bisphosphate ($PI[4,5]P_2$), in these processes. Here we have identified and functionally characterized a conserved binding site for $PI(4,5)P_2$ within μ2-adaptin, the medium chain of the clathrin adaptor complex AP-2. Mutant μ2 lacking a cluster of conserved lysine residues fails to bind $PI(4,5)P_2$ and to compete the recruitment of native clathrin/AP-2 to $PI(4,5)P_2$-containing liposomes or to presynaptic membranes. Moreover, we show that expression of mutant μ2 inhibits receptor-mediated endocytosis in living cells. We suggest that $PI(4,5)P_2$ binding to μ2-adaptin regulates clathrin-mediated endocytosis and thereby may contribute to structurally linking cargo recognition to coat formation.

## Introduction

Clathrin-mediated endocytosis is a vesicular transport process by which eukaryotic cells take up nutrients, internalize growth factor receptors (Mellman, 1996; Marsh and McMahon, 1999), and recycle synaptic vesicles after exocytotic activity (Hannah et al., 1999; Brodin et al., 2000; Slepnev and De Camilli, 2000; Jarousse and Kelly, 2001). Clathrin is the major structural component of coated pits (Hirst and Robinson, 1998; Kirchhausen, 2000; Robinson and Bonifacino, 2001) that coassembles with the heterotetrameric adaptor complex AP-2 and the monomeric coat protein AP180/CALM into clathrin-coated pits (Marsh and McMahon, 1999; Kirchhausen, 2000). This process is aided by several accessory proteins including amphiphysin, eps15, syndapin, endophilin, intersectin, and epsin (for reviews see Brodin et al., 2000; Slepnev and De Camilli, 2000). The AP-2 adaptor complex (composed of four subunits, α, β2, μ2, and σ2) executes two key functions in the initial stages of clathrin-coated pit nucleation: it recruits clathrin to the membrane and, via its μ2 subunit, selects specific cargo proteins (Robinson and Bonifacino, 2001). A crucial question is how AP-2 assembly and cargo recognition are regulated at endocytotic "hot spots" at the plasmalemma. Phosphoinositide lipids, in particular phosphatidylinositol (4,5)-bisphosphate ($PI[4,5]P_2$),* can directly interact with several endocytotic proteins, including α-adaptin (Gaidarov and Keen, 1999), thereby facilitating clathrin-mediated endocytosis (Jost et al., 1998; Arneson et al., 1999; Cremona et al., 1999; Wenk et al., 2001). Here we report on the identification and characterization of a novel phosphoinositide binding site within μ2-adaptin.

## Results and discussion

The endocytotic proteins α-adaptin, AP180, and epsin have been shown to interact with phosphoinositides via clusters of basic residues (Gaidarov and Keen, 1999; Ford et al., 2001; Itoh et al., 2001; Mao et al., 2001). Because μ2-adaptin harbors a major membrane binding site within AP-2 (Page and Robinson, 1995), we analyzed the primary sequence of μ2-

Address correspondence to Volker Haucke, Zentrum für Biochemie and Molekulare Zellbiologie, Department of Biochemistry II, University of Göttingen, Humboldtalle 23, D-37073 Göttingen, Germany. Tel.: 49-55-139-9854. Fax: 49-551-39 1-2198. E-mail: vhaucke@gwdg.de

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*Abbreviations used in this paper: HA, hemagglutinin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; P, phosphate; $P_2$, bisphosphate; $P_3$, trisphosphate; PI, phosphatidylinositol.

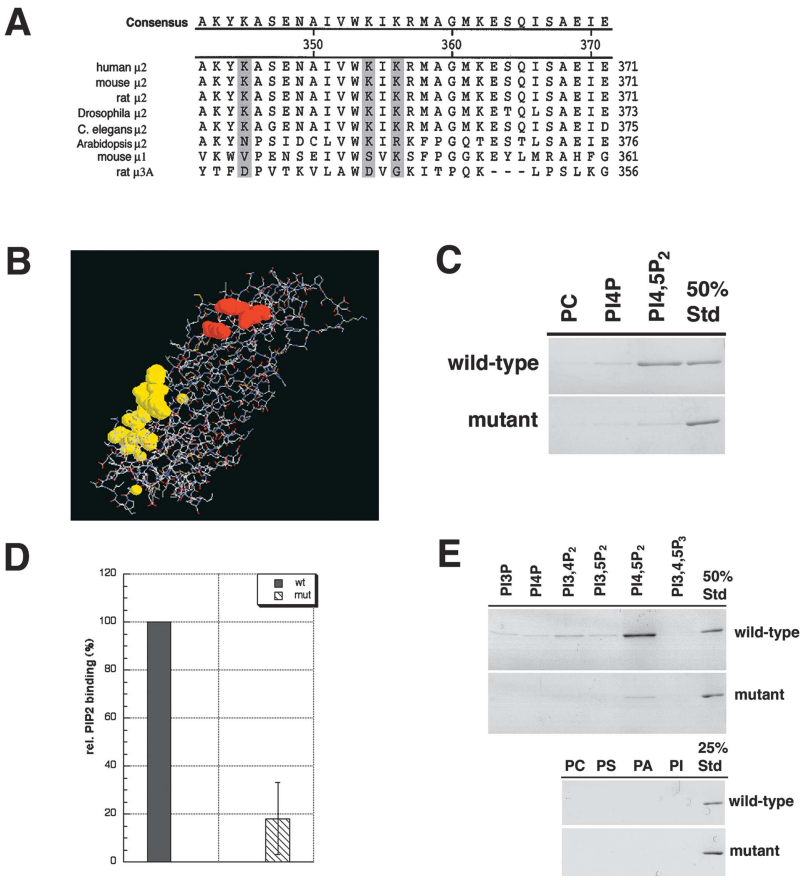


Figure 1. **Phosphoinositide binding of μ2-adaptin.** (A) Sequence alignment of μ-adaptins from various species. The conserved lysine residues within the putative phosphoinositide binding site are shaded. (B) Structural model of μ2 (aa 157–435) complexed with a tyrosine-based endocytosis signal (yellow) (modified from Owen and Evans, 1998). The three conserved lysine residues (red) form a basic patch at the surface of subdomain B. (C) Purified wild-type or mutant μ2 (2 μg) were incubated with liposomes containing 10% PC, PI(4)P, or PI(4,5)$P_2$. After reisolation of the liposomes, samples were analyzed by SDS-PAGE and staining with Coomassie blue. 50% Std, 50% of the protein sample added to the assay. (D) Quantification of the binding of wild-type or mutant μ2 to PI(4,5) $P_2$-containing liposomes from three independent experiments and plotted as mean ± SE. (E) Binding of μ2 to liposomes containing 10% of the indicated lipid. PS, phosphatidylserine; PA, phosphatidic acid. The experiment was performed as described in A.

adaptins from various species. Human, mouse, rat, fly, and nematode μ2-adaptins all shared a common sequence motif containing several conserved lysine residues reminiscent of the phosphoinositide binding site of AP-180 (Ford et al., 2001; Mao et al., 2001) (Fig. 1 A). Medium chains of AP complexes targeted to intracellular locations other than the plasma membrane, such as μ1- or μ3-adaptins, did not contain the putative phosphoinositide binding motif. This lysine cluster represents a surface-exposed positively charged patch within subdomain B of the crystallized cargo recognition domain of μ2 (Owen and Evans, 1998; Nesterov et al., 1999) (Fig. 1 B). To test whether μ2 can interact with phosphoinositides, we purified recombinant wild-type μ2 (aa 157–435) or a mutant in which three conserved lysine residues (K345, K354, and K356) had been replaced by glutamates (Fig. 1 C). We then prepared unilamellar liposomes composed of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) supplemented with 10% PI(4,5)$P_2$, PI (4)-phosphate (PI[4]P), or PC and assayed the ability of μ2 to interact with the liposomal membrane. Wild-type μ2 cosedimented only with PI(4,5)$P_2$-containing liposomes but not with PI(4)P or PC. The μ2 mutant (KKK–EEE) displayed a greatly reduced affinity for PI(4,5)$P_2$ (Fig. 1, C and D). Among different phosphoinositides, the strongest interaction was seen with PI(4,5)$P_2$, whereas both PI(3,5)$P_2$ and PI(3,4)$P_2$ were less effective in recruiting μ2. To our surprise, no specific interaction with PI (3,4,5)-trisphosphate (PI[3,4,5]$P_3$) or any other lipid tested was detectable (Fig. 1 E).

To see whether the mutant protein had retained the structural characteristics of wild-type μ2, we used limited proteolysis. Incubation of wild-type μ2 with increasing concentrations of trypsin generated two protease-resistant fragments, f1 and f2, similar to those seen with μ2 incorporated into AP-2 complexes (Aguilar et al., 1997). The mutant protein yielded identical proteolytic products upon limited digestion (Fig. 2 A), suggesting that it had retained native folding characteristics. An important functional property of μ2-adaptin is to recognize endocytosis signals of transmembrane cargo receptors (Mellman, 1996; Marsh and McMahon, 1999; Robinson and Bonifacino, 2001). We therefore analyzed the ability of the mutant protein to interact with tyrosine-based endocytosis signals. To this aim, we coupled peptides harboring the tyrosine-based endocytosis signal YQRL or a nonfunctional variant (AQRL) to beads (Rapoport et al., 1997) and assayed the ability of wild-type and mutant μ2 to interact specifically with these motifs. Both wild-type and mutant μ2 displayed an equal ability to recognize the functional YQRL peptide, whereas both of them failed to bind to the mutant AQRL variant (Fig. 2, B and C). Thus, mutation of the triple lysine cluster does not impair the ability of μ2 to recognize tyrosine-based endocytosis signals, which is in agreement with structural studies (Owen and Evans, 1998).

Clathrin/AP-2–coated pits can assemble efficiently on liposomal membranes (Takei et al., 1998). We therefore investigated whether purified μ2 could compete with clathrin/AP-2 recruitment to liposomes. We incubated liposomes

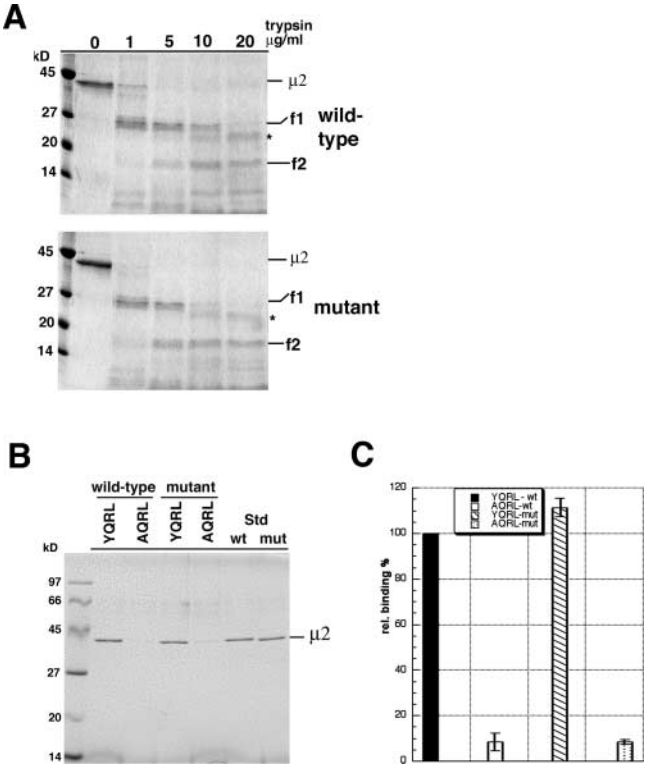


Figure 2. **Effects of the KKK–EEE mutation on the interaction of μ2 with tyrosine-based endocytosis signals and synaptotagmin.** (A) μ2 (157–435; 2 μg) was incubated with the indicated concentrations of trypsin for 15 min at RT. Samples were analyzed by 12% SDS-PAGE and staining with Coomassie blue. The asterisk denotes trypsin. (B) Mutant and wild-type μ2 (157–435; 2 μg) were incubated for 1 h at 4°C with peptides bearing the tyrosine-based endocytosis motif of TGN38 (YQRL) or its AQRL mutant immobilized on beads. Beads were reisolated, washed, and analyzed by SDS-PAGE and staining with Coomassie blue. Std, 50% of the protein added to the assay. (C) Quantification of the results shown in B. Data from three independent experiments were analyzed and plotted as mean ± SE. Binding of wild-type μ2 to the YQRL peptide was set as 100%.

containing 10% PI(4,5)$P_2$ with rat brain cytosol and ATP/GTPγS, conditions under which clathrin-coated pits are abundantly formed in vitro (Takei et al., 1996, 1998), in the presence or absence of purified μ2. After reisolation, clathrin/AP-2 association of the liposomes was analyzed by SDS-PAGE and staining with Ponceau S (Fig. 3 A) and immunoblotting (Fig. 3 B). Wild-type μ2 inhibited the recruitment of both clathrin and AP-2 to PI(4,5)$P_2$-containing liposomes, whereas the mutant version was an inefficient competitor (Fig. 3, A and B). Tubulin–liposome association was not affected. Inhibition was dose dependent, requiring low micromolar concentrations of μ2 (Fig. 3, C and D). This suggests that binding of PI(4,5)$P_2$ to μ2-adaptin may contribute to clathrin/AP-2 association with liposomes.

Next we analyzed whether PI(4,5)$P_2$ binding to μ2 might facilitate clathrin/AP-2 recruitment to native membranes. We first investigated the effect of neomycin, a drug that sequesters PI(4,5)$P_2$, on adaptor recruitment to isolated presynaptic LP2 membranes (Takei et al., 1996). In agreement with earlier observations, we found that neomycin inhibited clathrin/AP-2 recruitment (unpublished data; West et al., 1997; Jost et al., 1998). Likewise, neomycin partially inhibited the association of μ2 with synaptic membranes (Fig. 4 A). Recruitment of μ2 to LP2 membranes could also be inhibited by adding phospholipase Cδ1, an enzyme that specifically cleaves PI(4,5)$P_2$ (Fig. 4 B). These data suggest that phosphoinositides may aid targeting of μ2 to synaptic membranes. When we compared the ability of wild-type or mutant μ2 to bind to LP2 membranes, we noticed that only the wild-type, not the mutant, protein became efficiently recruited to the membrane (Fig. 4 C). We then incubated synaptosomal LP2 membranes with cytosol and ATP plus GTPγS in the presence of wild-type or mutant μ2. We found that only wild-type μ2, not its mutated counterpart, could effectively compete clathrin/AP-2 recruitment (Fig. 4, D and E), whereas membrane binding of hsc70 was not affected. An intact PI(4,5)$P_2$ binding site therefore is required for the ability of μ2-adaptin to compete clathrin/AP-2 recruitment to the plasma membrane.

Finally, we were interested to see whether mutant μ2 defective for PI(4,5)$P_2$ binding and incorporated into AP-2 complexes would affect AP-2 localization and receptor-mediated endocytosis in living cells. To this aim, we transiently expressed epitope-tagged versions of full-length wild-type or mutant μ2 bearing an internal hemagglutinin (HA) epitope. In agreement with previous studies (Nesterov et al., 1999), we found that both μ2 variants were incorporated into AP-2 complexes, as judged by specific coimmunoprecipitation of α-adaptin with HA–μ2 (Fig. 5 A). Whereas a large fraction of the expressed wild-type HA–μ2 was associated with membranes, most of the mutant version localized to the soluble fraction (Fig. 5 B). At low expression levels, both μ2 variants displayed a punctate distribution that colocalized with endogenous α-adaptin. High-level expression of mutant, but not wild-type, HA–μ2 resulted in a decreased association of α-adaptin with coated pits (Fig. 5 C). These data suggest that phosphoinositide binding to μ2-adaptin contributes to localizing AP-2 to clathrin-coated pits in vivo.

Cells expressing HA-tagged μ2 were then analyzed for their ability to internalize extracellularly added Texas red–labeled transferrin or EGF. Whereas cells expressing wild-type μ2 could endocytose transferrin normally (Fig. 5 D; Nesterov et al., 1999), cells expressing the PI(4,5)$P_2$ binding–defective mutant displayed a reduced ability to accumulate transferrin within recycling endosomes (Fig. 5 D). This defect was similar to that seen for a mutant of μ2 in which W421 was changed to alanine (unpublished data), a mutation known to impair the interaction of μ2 with the transferrin receptor (Owen and Evans, 1998; Nesterov et al., 1999). Similarly, transfected cells expressing mutant HA–μ2 were also less capable of internalizing Texas red–labeled EGF (Fig. 5 E). We conclude that the PI(4,5)$P_2$ binding site within μ2-adaptin facilitates clathrin-mediated endocytosis in living cells.

In the present study, we have identified and functionally characterized a PI(4,5)$P_2$ binding site within the μ2 subunit of AP-2. Structurally, the phosphoinositide binding motif within μ2 resembles the basic fingers found in AP180 (Ford et al., 2001; Mao et al., 2001), with several conserved lysine residues forming a surface-exposed posi-

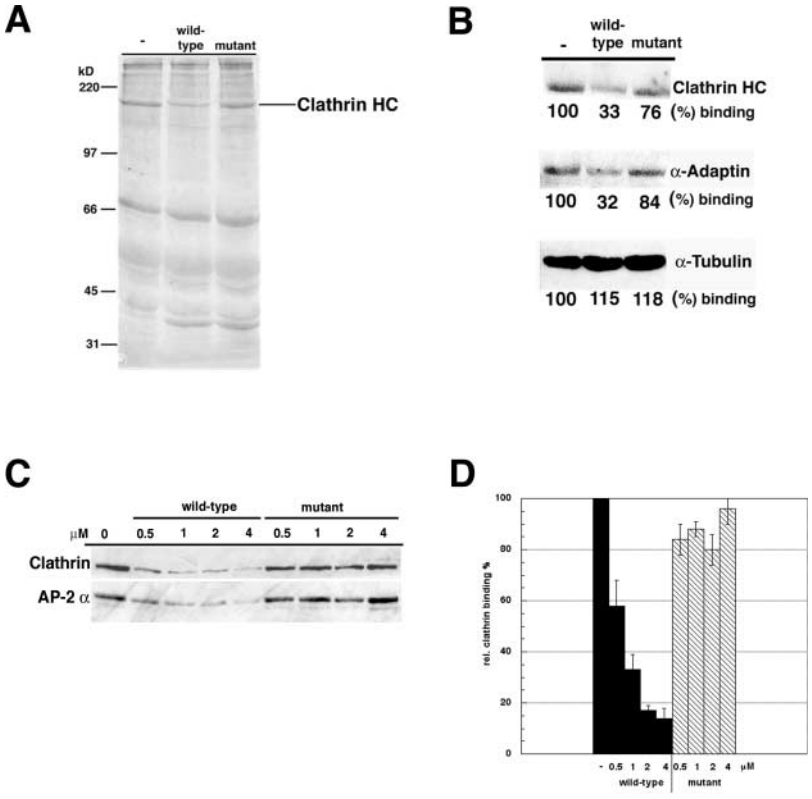


Figure 3. **Recruitment of clathrin/AP-2 to PI(4,5)$P_2$-containing liposomes.** (A and B) PI(4,5)$P_2$-containing liposomes were incubated with cytosol, ATP, GTPγS, and 4 μM wild-type or mutant μ2 (157–435). Liposomes were reisolated, washed, and analyzed by SDS-PAGE and staining with Ponceau S (A) or immunoblotting (B). Bands were visualized with $^{125}$I-protein A and quantified by phosphoimage analysis. (C) Dose dependence of μ2-mediated inhibition of clathrin/AP-2 recruitment to liposomes. The experiment was done as described in A, using the indicated concentrations of wild-type or mutant μ2. (D) Quantification of clathrin recruitment onto liposomes in the presence of different concentrations of μ2. Data are plotted as mean (±SE) from several experiments. The amount of clathrin recruited to liposomes in the absence of added μ2 was taken as 100%.

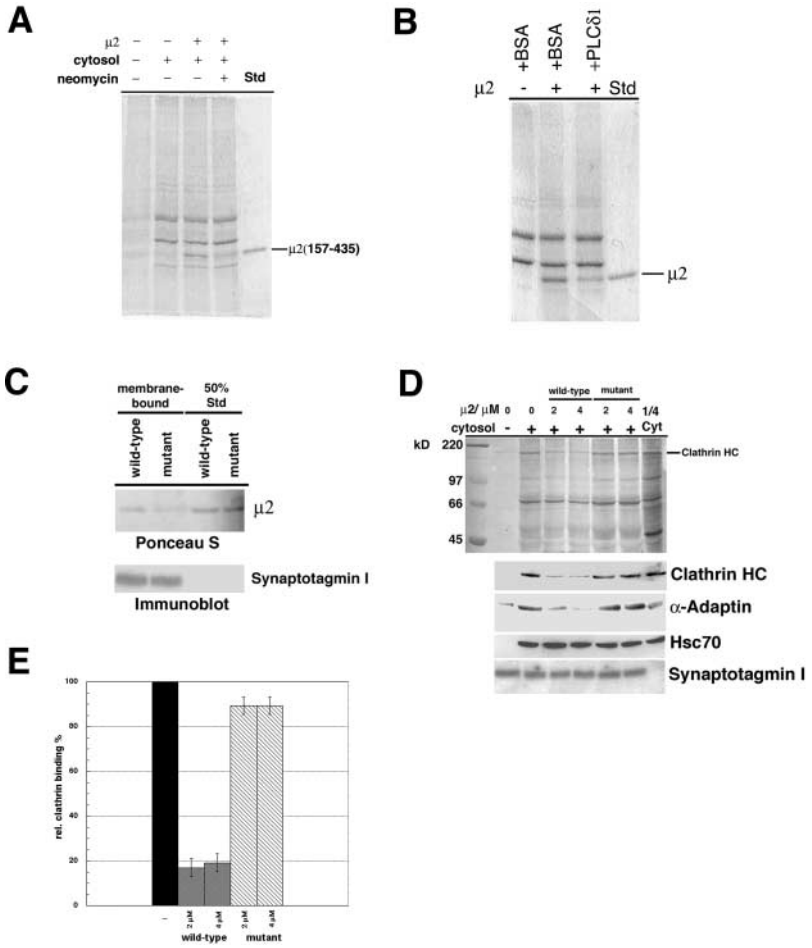


Figure 4. **Recruitment of clathrin/AP-2 and μ2 to synaptic LP2 membranes.** (A) Membrane association of μ2 is inhibited by neomycin. Carbonate-washed LP2 membranes (10 μg) were incubated with cytosol (0.4 mg/ml), ATP, GTPγS, and μ2 (157–435; 1.5 μg) in the presence or absence of 2 mM neomycin. LP2 membranes were reisolated, washed, and analyzed by Western blotting and staining with Ponceau S. Std, 50% of the μ2 added to the assay. (B) Membrane association of μ2 is inhibited by phospholipase Cδ1. Recruitment of μ2 was assayed as described in A in the presence or absence of 5 μg purified phospholipase Cδ1 or BSA. (C) Membrane recruitment of μ2 requires an intact PI(4,5)$P_2$-binding site. Recruitment of wild-type or mutant μ2 was analyzed as in A, except that the samples were analyzed by staining with Ponceau S to detect bound μ2 and immunoblotting for synaptotagmin I as a membrane marker. (D) Clathrin/AP-2 recruitment to LP2 membranes can be competed by wild-type but not KKK–EEE mutant μ2. LP2 membranes (20 μg) were incubated with cytosol, ATP, GTPγS, and 2 or 4 μM of wild-type or mutant μ2 (157–435). Membranes were reisolated, washed, and analyzed by staining with Ponceau S (top) or immunoblotting (bottom) for clathrin heavy chain (HC), α-adaptin, hsc70, and synaptotagmin I. 1/4 cyt, 25% of the cytosol used in the experiment. (E) Quantification of clathrin recruitment as shown in D. The amount of clathrin recruited to LP2 in the absence of μ2 was taken as 100%. Data are plotted as mean (±SE) from three independent experiments.

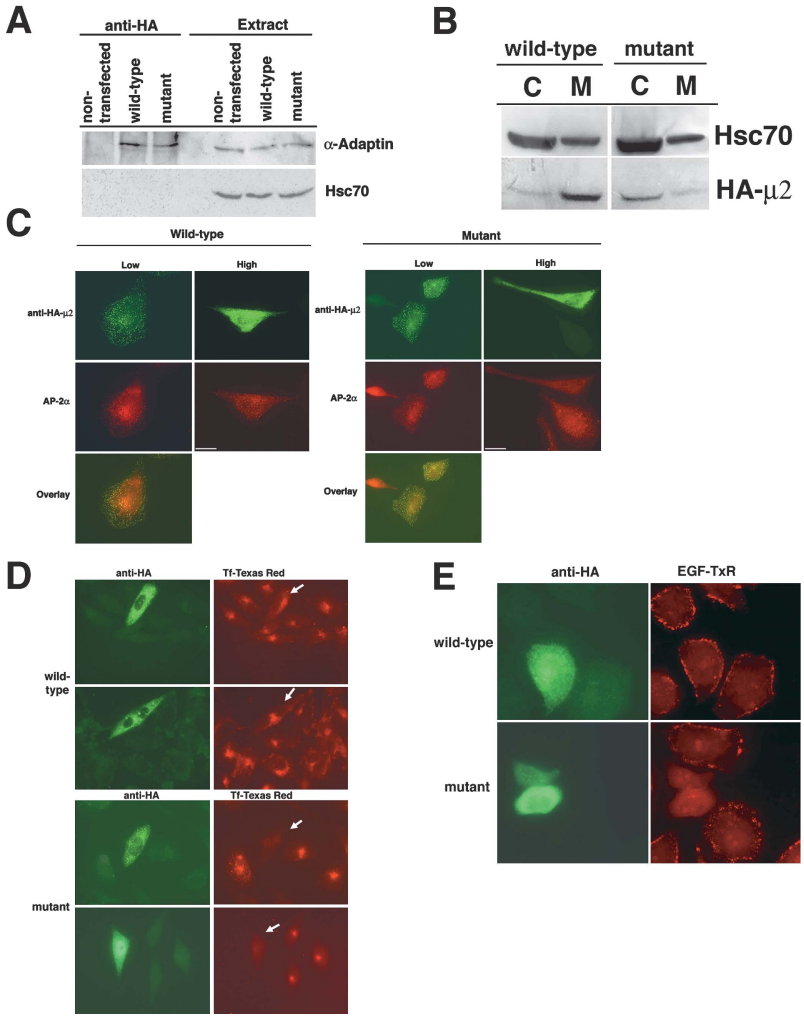


Figure 5. **Expression of KKK–EEE mutant μ2 inhibits clathrin-mediated endocytosis in CHO cells.** (A) CHO cells ($10 \times 10^6$) transiently transfected with HA-tagged wild-type or mutant μ2 were lysed and subjected to immunoprecipitations with monoclonal antibodies against the HA tag. Samples were analyzed by SDS-PAGE and immunoblotting for α-adaptin and hsc70. Extract, 10% of the total extracted proteins used for the experiment. (B) Transfected CHO cells (as in A) were fractionated into membrane (M) and cytosol (C). Samples were analyzed by SDS-PAGE and immunoblotting for HA-tagged μ2 or hsc70. (C) HA-tagged wild-type or mutant μ2 were transiently expressed in CHO cells. 48 h after transfection, cells were methanol fixed and immunostained with antibodies against α-adaptin or HA. Bar, 10 μm. (D) HA-tagged wild-type or mutant μ2 was transiently expressed in CHO cells (see C) and analyzed for their ability to internalize Texas red–labeled transferrin (2.5 μg/ml; 10 min at 37°C) by immunofluorescence microscopy. Transfected cells are indicated by an arrow. The results are representative of three independent transfection experiments in which 85% of the cells expressing elevated levels of mutant μ2 displayed strongly reduced transferrin uptake. (E) HA-tagged wild-type or mutant μ2 were transiently expressed in HeLa cells and analyzed for the ability to internalize Texas red–labeled EGF (2 μg/ml; 3 min at 37°C) by immunofluorescence microscopy.

tively charged patch. In contrast to α-adaptin, which preferentially recognizes $PIP_3$ over $PIP_2$, μ2 displays a high degree of specificity for $PI(4,5)P_2$. This suggests that AP-2 may interact with phosphoinositides via two independent sites within its α and μ2 subunits. One possibility is that phosphoinositide binding to both subunits synergistically triggers coated pit assembly. Alternatively, different stimuli, such as growth factor activation, could result in the formation of distinct species of PI lipids (Di Fiore and De Camilli, 2001; McPherson et al., 2001). These might then exert differential effects on AP-2 with regard to coated pit assembly or cargo recognition. Lastly, it is conceivable that $PI(4,5)P_2$ and $PI(3,4,5)P_3$ sequentially interact with μ2 and α-adaptin during progressive coated pit invagination (Kirchhausen, 2000; Slepnev and De Camilli, 2000) or upon cargo selection and transport. Support for the latter possibility comes from the observation that 3′-phosphoinositides, which are synthesized by a clathrin-activated kinase (Gaidarov et al., 2001), stimulate binding of tyrosine-based endocytosis signals to AP-2 (Rapoport et al., 1997). It is therefore conceivable that binding of 3′-phosphoinositides to AP-2 induces a conformational change that allows μ2 to interact more efficiently with cargo protein sorting signals at the membrane perhaps assisted by the concomitant association of μ2 with $PI(4,5)P_2$.

We favor the possibility that the temporally or spatially ordered interaction of several endocytotic proteins with $PI(4,5)P_2$ is required for clathrin-mediated endocytosis. This view is supported by the observation that the phosphoinositide binding sites within AP180/CALM (Ford et al., 2001; Mao et al., 2001), α-adaptin (Gaidarov and Keen, 1999), and μ2 (this study) apparently cannot substitute for one another. Although binding of phosphoinositides to α-adaptin may largely function in targeting AP-2 to the plasma membrane (Gaidarov and Keen, 1999), the association of μ2 with $PI(4,5)P_2$ could in addition help to stabilize AP-2 at sites of endocytosis or to reorient the molecule with respect to the membrane.

In conclusion, the results reported here support a model according to which the binding of phosphoinositides to μ2-adaptin, as well as to other endocytotic proteins, links the formation of clathrin/AP-2–coated pits with the selection of cargo proteins.

## Materials and methods

### Antibodies and lipids

All lipids used were from Sigma-Aldrich or Calbiochem. Monoclonal antibodies against clathrin (TD.1) and synaptotagmin I (Cl41.1) were gifts from Pietro De Camilli (Yale University, New Haven, CT) and Reinhard Jahn (Max-Planck Institute). Anti–α-adaptin, α-tubulin, and anti-HA antibodies were from Affinity BioReagents, Inc. and Sigma-Aldrich, respectively.

### Molecular biology procedures

Constructs encoding full-length μ2-adaptin bearing an internal HA epitope tag (YPYDVDYA) between aa 236 and 237 (Nesterov et al., 1999) were generated by PCR (plasmid DNA was a gift from J.S. Bonifacino, National Institutes of Health, Bethesda, MD), subcloned into pcDNA3, and verified by DNA sequencing. Hexahistidine-tagged wild-type or mutant versions of μ2-adaptin (aa 157–435) were made by PCR, subcloned into pET28a (Novagen Inc.), and verified by DNA sequencing. Standard techniques were used for preparation of plasmid and genomic DNA, restriction analysis, PCR, and cloning of DNA fragments.

### Generation of liposomes

Unilamellar liposomes were made as previously described (Takei et al., 1998), by using a mixture of defined lipids (70% PC, 20% PE, plus 10% variable lipid [wt/wt]). Liposome recovery was followed by tagging with the lipophilic dye 1,6-diphenyl-1,3,5-hexatriene ($\lambda_{EX}$ = 360 nm; $\lambda_{EM}$ = 430 nm) on a fluorimeter after reisolation of the liposomes.

### Protein recruitment to membranes and liposomes

For biochemical analysis of protein recruitment onto synaptic LP2 membranes, published procedures were used (Takei et al., 1996, 1998). Protein binding to liposomes (Fig. 1) was done as follows: 100 μg liposomes was incubated for 30 min at RT with 2 μg purified μ2 (aa 157–435) in cytosolic buffer. Liposomes were reisolated by sedimentation, washed extensively, and analyzed by SDS-PAGE and staining with Coomassie blue. For the experiments shown in Fig. 3, liposomes (0.4 mg/ml) were incubated with rat brain cytosol (3 mg/ml) in the presence of 2 mM ATP, 200 μM GTPγS, and purified μ2 (aa 157–435) for 10 min at 37°C, chilled on ice, and reisolated as described above.

### Transfection experiments and endocytosis assay

CHO (Fig. 5, A–D) or HeLa (Fig. 5 E) cells were transfected with plasmids encoding internally HA-tagged μ2-adaptin (wild type or mutant) with Lipofectamine 2000 (GIBCO BRL). 48 h after transfection, cells were washed and incubated for 2–3 h in serum-free medium. Texas red–labeled transferrin (2.5 μg/ml) or EGF (2 μg/ml) were added and cells were allowed to internalize the probe for 10 or 3 min at 37°C, respectively. Cells were acid washed, fixed, and processed for indirect immunofluorescence microscopy.

For biochemical fractionation studies, cells were harvested 40 h after transfection by scraping into isotonic buffered sucrose (10 mM Hepes, 320 mM sucrose, pH 7.4, 1 mM PMSF). Cells were homogenized using an EMBL cell cracker and centrifuged at 1,500 *g*. The supernatant was separated into membrane and soluble fractions by centrifugation for 30 min at 12,000 *g*, and samples were precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE.

### Miscellaneous

Rat brain cytosol and LP2 synaptic membrane preparations have been described previously (Takei et al., 1996; 1998). Hexahistidine-tagged fusion proteins were purified from CHAPS (2%)-lysed *Escherichia coli* detergent extracts according to the manufacturer's instructions. Bacterially expressed histidine-tagged phospholipase Cδ1 was purified according to Bromann et al. (1997). Standard procedures were used for indirect immunofluorescence, SDS-PAGE, and immunoblotting.

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