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Dual interaction of synaptotagmin with μ2- and α-adaptin facilitates clathrin-coated pit nucleation

Volker Haucke[1], Markus R.Wenk, Edwin R.Chapman[2], Khashayar Farsad and Pietro De Camilli[3]

Department of Cell Biology and Howard Hughes Medical Institute, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06510 and [2]Department of Physiology, University of Wisconsin, 1300 University Avenue, Madison, WI 53706, USA

[1]Present address: Zentrum für Biochemie und Molekulare Zellbiologie, Georg-August-Universität Göttingen, Humboldtallee 23, D-37073 Göttingen, Germany

[3]Corresponding author
e-mail: pietro.decamilli@yale.edu

The synaptic vesicle protein synaptotagmin was proposed to act as a major docking site for the recruitment of clathrin coats implicated in endocytosis, including the recycling of synaptic vesicles. We show here that the C2B domain of synaptotagmin binds μ2- and α-adaptin, two of the four subunits of the endocytic adaptor complex AP-2. μ2 represents the major interacting subunit of AP-2 within this complex. Its binding to synaptotagmin is mediated by a site in subdomain B that is distinct from the binding site for tyrosine-based sorting motifs located in subdomain A. The presence of the C2B domain of synaptotagmin at the surface of liposomes enhances the recruitment of AP-2 and clathrin. Conversely, perturbation of the interaction between synaptotagmin and AP-2 by synprint, the cytoplasmic synaptotagmin-binding domain of N-type calcium channels, inhibits transferrin internalization in living cells. We conclude that a dual interaction of synaptotagmin with the clathrin adaptor AP-2 plays a key physiological role in the nucleation of endocytic clathrin-coated pits.

Keywords: AP-2 adaptor/clathrin/endocytosis/synaptic vesicles/synaptotagmin

Introduction

Membrane transport in eukaryotic cells is effected largely by intracellular vesicles that bud from donor membranes to transport cargo proteins selectively. One of the most thoroughly characterized budding reactions involves clathrin coats. Clathrin-coated vesicles mediate a variety of intracellular transport steps such as endocytosis, transport of proteins from the *trans*-Golgi network to the endosomal/lysosomal system and budding from endosomes (Mellman, 1996; Stoorvogel *et al*., 1996; Kirchhausen *et al*., 1997). A specialized form of clathrin-mediated endocytosis takes place at the nerve terminal, where clathrin-coated vesicles are involved in the recycling of synaptic vesicles (Heuser and Reese, 1973; Cremona and De Camilli, 1997). Nerve terminal clathrin coats comprise clathrin, the adaptor complex AP-2 (a heterotetramer composed of α, β2, μ2 and σ2 subunits) and the protein AP180 (Morris *et al*., 1993; Ye and Lafer, 1995; Hirst and Robinson, 1998). Coated vesicle formation in the presynaptic compartment is believed to start with the recruitment of the AP-2 adaptor complex at or near sites of exocytosis (Gad *et al*., 1998), where synaptic vesicle proteins destined to re-internalize are localized. Focal accumulation of specific phospholipids, including phosphoinositides (Cremona *et al*., 1999; Gaidarov and Keen, 1999), may also contribute to binding of adaptors to the membrane. AP-2 then recruits clathrin and the resulting coated pit subsequently matures into a deeply invaginated coated bud (Heuser, 1989; Marsh and McMahon, 1999). Finally, a free clathrin-coated vesicle is pinched off in a process that requires the function of the GTPase dynamin (Hinshaw and Schmid, 1995; Takei *et al*., 1995; Sever *et al*., 1999). Additional factors such as amphiphysin (Shupliakov *et al*., 1997) and endophilin I (Ringstad *et al*., 1999; Schmidt *et al*., 1999) also participate in this fission reaction.

Clathrin-coated bud formation at the plasma membrane involves interactions of the AP-2 adaptor complex with both membrane proteins (Bonifacino and Dell'Angelica, 1999; Haucke and De Camilli, 1999) and lipids (West *et al*., 1997; Takei *et al*., 1998; Arneson *et al*., 1999; Cremona *et al*., 1999; Gaidarov and Keen, 1999). Although the interaction of the AP-2 complex with lipids is sufficient to drive coated pit assembly on liposomes (Takei *et al*., 1998), it is clear that coated vesicle formation at the synapse is highly regulated *in vivo* and tightly coupled to the exocytic fusion of synaptic vesicles with the nerve terminal plasma membrane (Heuser and Reese, 1973; Gad *et al*., 1998). Synaptotagmin, an AP-2-binding protein of synaptic vesicle membranes, may function as a high affinity docking site for AP-2 and thus serve as a link between the exocytic and endocytic limbs of the synaptic vesicle cycle (Mahaffey *et al*., 1990; Zhang *et al*., 1994). *In vivo* studies in nematodes (Jorgensen *et al*., 1995) and the squid giant synapse (Fukuda *et al*., 1995) support this model. Isoforms of this protein are present in synaptic vesicles, the presynaptic plasma membrane (Butz *et al*., 1999) and in non-neuronal cells (Li *et al*., 1995; Haucke and De Camilli, 1999; Martinez *et al*., 2000). We have shown recently that tyrosine-based endocytic motifs of clathrin-coated vesicle membrane proteins can stimulate AP-2 binding to the C2B domain of synaptotagmin, suggesting a possible mechanism for coupling coated pit nucleation to the selection of a variety of cargo proteins during endocytosis (Haucke and De Camilli, 1999).

While the AP-2-binding site within the C2B domain of synaptotagmin has been mapped (Chapman *et al*., 1998), no such information is available for the corresponding interaction site within the heterotetrameric AP-2 complex.

In the present study, we have mapped the synaptotagmin-binding site within AP-2 and have investigated further the functional importance of this interaction *in vitro* and in living cells.

Results

Synaptotagmin interacts with the μ2 and α subunits of AP-2

To identify the AP-2 subunits that interact with the C2B domain of synaptotagmin, we studied the binding of *in vitro* translated subunits to fusion proteins comprising glutathione *S*-transferase (GST) fused to either the C2A or the C2B domains of synaptotagmin I (see also Haucke and De Camilli, 1999). All four subunits of AP-2 were synthesized individually by coupled transcription–translation *in vitro* in the presence of ^{35}S-labeled methionine, and incubated with GST fusion proteins (Figure 1A). Bound proteins were then detected by SDS–PAGE and autoradiography. As shown in Figure 1B, μ2-adaptin strongly bound to the C2B domain fusion protein, while neither β2 nor σ2 showed any detectable interaction. A small but reproducible fraction of α-adaptin also bound to the C2B domain fusion protein. None of the subunits bound to glutathione–Sepharose beads alone or to immobilized GST–C2A (Figure 1B). When the same experiment was performed with a protein comprising GST fused to amino acids 1–96 (the entire cytoplasmic domain) of rat VAMP/synaptobrevin I, no binding of any of the AP-2 subunits to this protein was detectable (Figure 1C). In control experiments, we found that only the μ2 subunit could bind specifically to tyrosine-based endocytic motif peptides immobilized on beads, as expected (not shown).

To corroborate these data further, we carried out binding experiments using disassembled subunits of AP-2 obtained from urea-denatured purified clathrin coats (Takei *et al*., 1998). When the AP-2 present in the clathrin coat protein fraction was first disassembled by denaturation with 8 M urea (Prasad and Keen, 1991) and then presented to the GST–C2B fusion protein after dilution with buffer, a signal for μ2 was detectable in the affinity-purified material. A small fraction of α-adaptin also seemed to be capable of binding to GST–C2B after denaturation. In contrast, the β2 subunit did not bind (Figure 1D). Incubation of the native clathrin coat protein fraction with the GST–C2B domain fusion protein resulted in the recovery of all AP-2 subunits in the affinity-purified material. None of these AP-2 subunits were detectable in samples containing a synaptotagmin I C2A domain fusion protein incubated with native (Figure 1D) or urea-denatured (not shown) coat proteins.

To confirm the specificity of the described interactions, we took advantage of a mutant form of synaptotagmin I in which two lysines (K326 and K327) had been mutated to alanines. This mutant failed to oligomerize and showed reduced binding to AP-2 (Chapman *et al*., 1998). We compared the ability of immobilized GST fusion proteins comprising both C2 domains of wild-type and mutant synaptotagmin I to interact with either native AP-2 or μ2-adaptin *in vitro*. As expected, the wild-type protein bound efficiently to native AP-2 (revealed by the presence of β2-adaptin in the bound material), but not to AP-1 (indicated by the absence of its β1 subunit in the bound material) (Figure 1D, top panel) or to *in vitro* translated μ2-adaptin (Figure 1D, bottom panel). In contrast, decreased binding of the K326A,K327A mutant protein to both native AP-2 and μ2-adaptin was observed (Figure 1D).

The synprint peptide inhibits the interaction of synaptotagmin with the μ2 and α subunits of AP-2

A peptide corresponding to the intracellular loop between the second and third transmembrane segments of the α1 subunit of N-type calcium channels, synprint, has recently been reported to inhibit specifically the AP-2–synaptotagmin interaction in direct binding assays *in vitro* (Chapman *et al*., 1998). The synprint peptide binds to a site within the C2B domain of synaptotagmin that appears to be critical for AP-2 binding (Figure 2A; see also Chapman *et al*., 1998) and oligomerization (Kim and Catterall, 1997; Sheng *et al.*, 1997; Chapman *et al*., 1998). As a further control for the specificity of our binding studies, we studied the effect of this peptide on the interaction of μ2- and α-adaptin with synaptotagmin. As shown in Figure 2B, binding of *in vitro* translated μ2- or α-adaptin to the GST–C2B domain fusion protein was reduced by the presence of synprint in the reaction mixture (Figure 2B).

Collectively, the data described above suggest that synaptotagmin specifically binds to AP-2 via a dual interaction of its C2B domain with μ2- and α-adaptin, with μ2 harboring the major interacting site.

Tyrosine-based endocytic motifs do not affect the μ2–synaptotagmin interaction

Tyrosine-based endocytic motifs that bind the μ2 subunit of AP-2 stimulate the interaction of the AP-2 holocomplex with synaptotagmin, presumably via a conformational change of the complex (Haucke and De Camilli, 1999). We tested whether a soluble 14mer peptide containing the endocytic motif YQRL (Haucke and De Camilli, 1999) had any effect on the binding of μ2 alone to GST–C2B. No effect was seen in the presence of either the YQRL peptide or its AQRL mutant under conditions where only ~20% of the added μ2 was associated with the fusion protein (Figure 2C). This suggests that AP-2 subunits other than μ2 are also required for the conformational change of the AP-2 complex that stimulates its interaction with synaptotagmin.

Mapping of the synaptotagmin-binding site within μ2-adaptin

Recently, the crystal structure of a rat μ2 fragment (amino acids 158–435) bound to a tyrosine-based endocytic motif peptide has been reported (Owen and Evans, 1998). This fragment is largely composed of β structures that are folded into two subdomains. Subdomain A, which comprises the N-terminal half of this fragment and its C-terminal region, harbors the binding site for tyrosine-based endocytic motifs (Owen and Evans, 1998). Subdomain B comprises the portion of the protein between these two amino acid stretches and corresponds approximately to amino acids 283–394 (Figure 3A).

We investigated the region within μ2 that contains the synaptotagmin-binding site. Towards this aim, various μ2 fragments were synthesized by coupled transcription–

translation *in vitro* (depicted schematically in Figure 3A) and their ability to bind the GST–synaptotagmin fusion proteins was determined. A fragment comprising amino acids 158–435 retained the ability to bind the C2B domain of synaptotagmin (Owen and Evans, 1998), while an N-terminal fragment comprising amino acids 1–157 did not bind. The most C-terminal residues of μ2 were dispensable for synaptotagmin binding, in spite of their requirement for efficient binding of tyrosine-based endocytic motifs (Owen and Evans, 1998; Nesterov *et al*., 1999) (construct 158–407 of Figure 3B). Furthermore, a fragment encompassing subdomain B (residues 283–394) bound GST–C2B. Fragment 158–282, corresponding to the N-terminal portion of subdomain A, did not bind either C2B or tyrosine-based motifs (Figure 3B), but was probably misfolded due to the critical contribution of C-terminal sequences to this domain (Figure 3A). We conclude that the binding sites of μ2 for tyrosine-based motifs and for synaptotagmin are distinct.

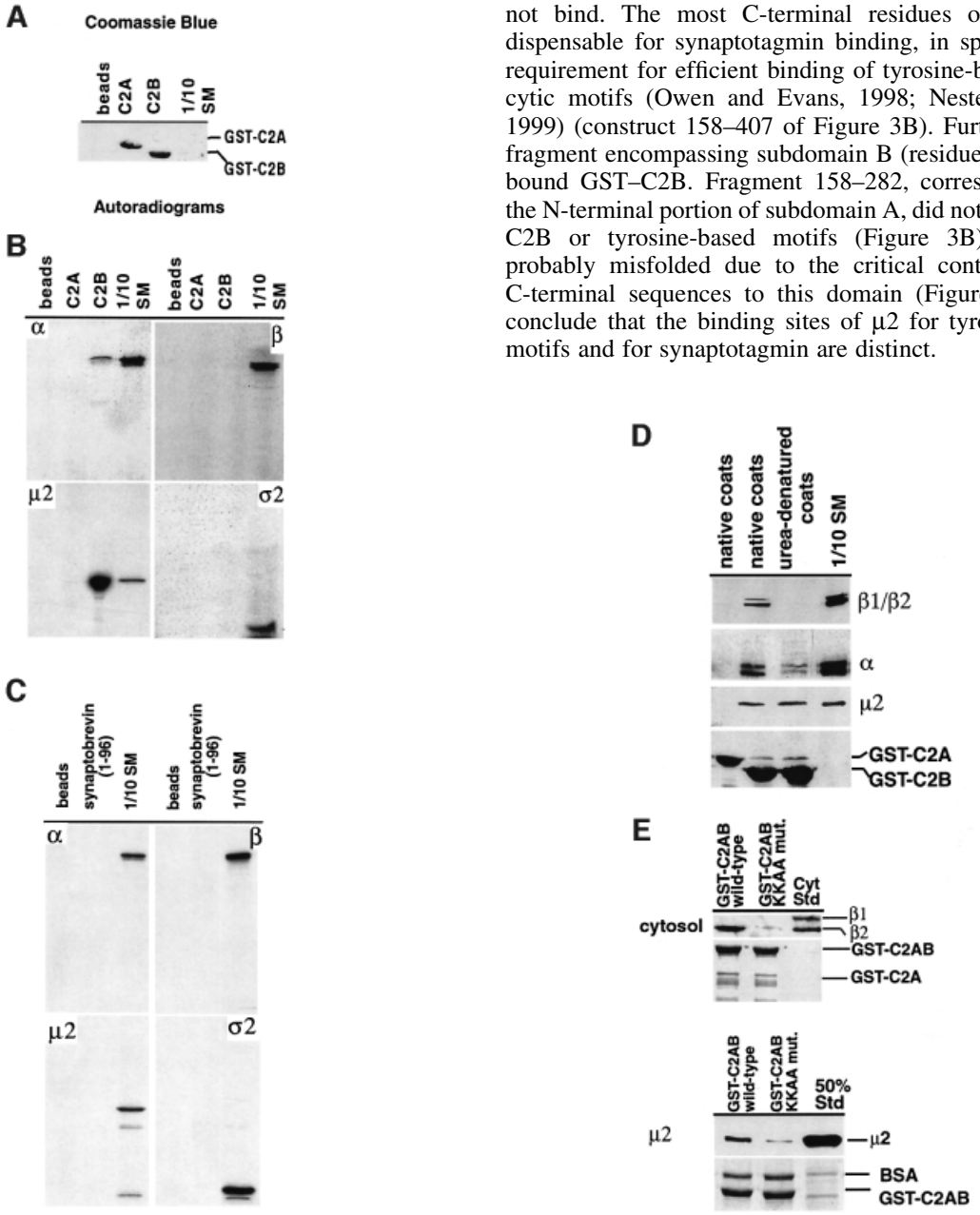

Fig. 1. Dual interaction of the μ2 and α subunits of AP-2 with synaptotagmin. (**A** and **B**) Specific interaction of the μ2 and α subunits of AP-2 with synaptotagmin. ^{35}S-labeled α-, β2-, μ2- and σ2-adaptins were synthesized by coupled transcription–translation *in vitro*. Post-ribosomal supernatants were then incubated with 20 μg/ml GST–synaptotagmin I C2 domain fusion proteins (C2A or C2B domains) or glutathione beads alone (beads) for 4 h at 4°C in buffer T, washed, eluted with sample buffer and analyzed by SDS–PAGE, staining with Coomassie blue (A) and autoradiography (B). 1/10 SM = 1/10 of the total amount of radiolabeled protein added to the assay. (**C**) None of the AP-2 subunits can bind to GST–synaptobrevin/VAMP. ^{35}S-labeled α-, β2-, μ2- and σ2-adaptins were synthesized by coupled transcription–translation *in vitro*, incubated with 20 μg/ml GST–synaptobrevin I fusion proteins (amino acids 1–96) or glutathione beads alone (beads), and analyzed as described in (B). 1/10 SM = 1/10 of the total amount of radiolabeled protein added to the assay. (**D**) Synaptotagmin binds the μ2 and α subunits of disassembled AP-2. Clathrin coat proteins (0.5 mg/ml) from bovine brain (Haucke and De Camilli, 1999) were denatured with 7.5 M urea for 15 min at room temperature and then diluted 5-fold into phosphate-buffered saline (PBS) containing 1% Triton X-100. Adaptor proteins were affinity purified by incubation with 20 μg/ml GST–synaptotagmin I C2 domain fusion proteins (C2A or C2B) for 3 h at 4°C immobilized on glutathione–Sepharose beads. Samples were washed extensively, eluted with sample buffer and analyzed by SDS–PAGE and immunoblotting for GST, α-, β- or μ2-adaptin. 1/10 SM = 1/10 of the total amount of coat proteins added to the assay. (**E**) A mutant of synaptotagmin defective for binding AP-2 or μ2-adaptin. Rat brain cytosol (5 mg/ml) or radiolabeled *in vitro* translated μ2-adaptin was incubated with 10 μg/ml GST–synaptotagmin I C2AB domain fusion proteins (wild-type or KKAA mutant) for 2 h at 4°C, washed, eluted with sample buffer and analyzed as described in (B) (bottom panel) or by SDS–PAGE followed by immunoblotting for β1/β2-adaptins (top panel). 50% Std = half of the total amount of radiolabeled μ2 protein added to the assay. Cyt Std = 100 μg of rat brain cytosol.

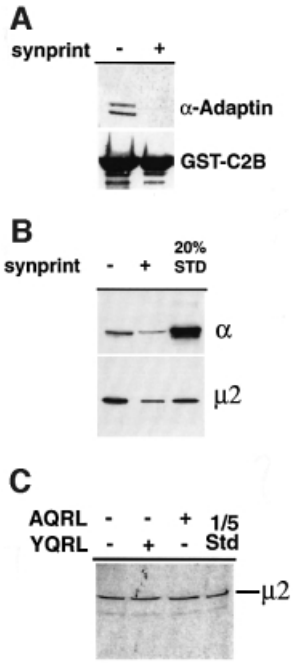

Fig. 2. Synprint, but not a tyrosine-based motif peptide, inhibits the interaction of synaptotagmin with AP-2 or μ2-adaptin. (**A**) Synprint blocks the interaction between AP-2 and synaptotagmin *in vitro*. GST–synaptotagmin I C2B (20 μg/ml) was incubated with rat brain cytosol (3 mg/ml) in the presence or absence of 0.2 mg/ml synprint peptide (Chapman *et al*., 1998) for 2 h at 4°C, washed extensively and eluted with SDS–PAGE sample buffer. The affinity-purified material was analyzed by SDS–PAGE and immunoblotting with antibodies against AP-2 and GST. (**B**) Synprint inhibits the interaction between synaptotagmin and μ2- or α-adaptin *in vitro*. Radiolabeled μ2- or α-adaptin was incubated with 10 μg/ml GST–synaptotagmin I fusion proteins in the presence or absence of 0.2 mg/ml synprint peptide and analyzed as described in the legend to Figure 1B. 20% STD = 1/5 of the total amount of radiolabeled protein added to the assay. (**C**) Tyrosine-based endocytic motif peptides do not influence the interaction of μ2 with synaptotagmin. Radiolabeled μ2-adaptin was incubated with 2 μg/ml GST–synaptotagmin I fusion proteins in the presence or absence of 300 mM YQRL- or AQRL-containing 14mer peptides and analyzed as described in the legend to Figure 1B. 1/5 Std = 1/5 of the total amount of radiolabeled protein added to the assay.

Since fragment 283–394 of μ2 is relatively rich in charged amino acids (Ohno *et al*., 1995) and since the interaction of μ2 with GST–C2B is salt sensitive (not shown), we inspected the primary structures of μ1-, μ2- and μ3-adaptins from various species for the presence of charged regions that are unique to μ2-adaptins and conserved among different species. We hypothesized that the major synaptotagmin-binding site within μ2 is contained in a charged region spanning β-strands 11–13 within the fragment encompassing amino acids 158–435 (Owen and Evans, 1998).

In order to address directly whether this region of μ2-adaptin was indeed important for efficient binding to synaptotagmin, we generated a double point mutant of μ2. In this construct, two residues conserved among all known μ2-adaptins, Tyr344 and Lys354, were changed to alanines. *In vitro* translated mutant μ2 bound less efficiently than wild-type μ2 to GST–C2B (Figure 4A). In contrast, the mutant μ2 retained its ability to interact with immobilized tyrosine-based endocytic motifs, suggesting that it is folded correctly (Figure 4B).

The triple lysine mutant of α-adaptin has a reduced affinity for synaptotagmin

We also tested the synaptotagmin-binding properties of a previously characterized mutant form of α-adaptin in which a lysine triad in the N-terminal part of the molecule is mutated to glutamines. AP-2 complexes harboring this mutant α-adaptin were shown previously to be defective in binding to phosphoinositides and in recruitment to the plasma membrane (Gaidarov and Keen, 1999). The ability of the *in vitro* translated α-adaptin mutant to bind the C2B domain of synaptotagmin was significantly reduced compared with the wild-type form of the protein (Figure 4C). Therefore, it remains to be seen whether the impaired plasma membrane recruitment of AP-2 complexes harboring this α-adaptin mutant *in vivo* (Gaidarov and Keen, 1999) is attributable mainly to impaired interactions with phosphoinositides, synaptotagmin or both.

Binding of α-adaptin and μ2 to synaptic membranes is facilitated by the interaction with synaptotagmin

Next we tested whether the described interactions of synaptotagmin with α-adaptin and μ2 are functionally important for the recruitment of these subunits to synaptic membranes. When each of the four radiolabeled *in vitro* translated AP-2 subunits were incubated with carbonate-washed synaptic LP2 membranes, only μ2- and α-adaptin bound to these membranes (Figure 5A). Membrane recruitment of both subunits was inhibited by the synprint peptide and may thus be mediated, at least in part, by interactions with synaptotagmin within the membrane (Figure 5B). Consistent with this interpretation, the double point mutant of μ2, which is defective in binding to synaptotagmin (see Figure 4), is also defective in binding to synaptic membranes (Figure 5C).

The presence of the synaptotagmin C2B domain at the surface of liposomes facilitates clathrin/AP-2 recruitment

Previous studies and the results presented above have shown that AP-2 binds the C2B domain of synaptotagmin. However, no evidence has been presented so far that the presence of synaptotagmin at the cytosol–membrane interface can actively facilitate clathrin/AP-2 recruitment. We tested this hypothesis using artificial liposomes (Matsuoka *et al*., 1998). Towards this aim, reduced glutathione was first coupled to phosphatidylethanolamine (PE) using a modified version of PE, *N*-MPB-PE, which contains a cysteine-reactive chemical cross-linker within its headgroup (Figure 6A). We then prepared liposomes from a crude brain lipid extract supplemented with 20% (w/w) glutathione-linked PE or unmodified PE and checked the ability of these liposomes to bind GST fusion proteins. As expected, the presence of glutathione greatly enhanced the binding of GST or GST–C2B to liposomes (Figure 6B). We then assayed the ability of liposomes containing similar amounts of either GST or the synaptotagmin GST–C2B fusion protein (Figure 6C) to recruit clathrin and AP-2 from a coat protein fraction (Takei *et al*., 1998). Liposomes containing GST–C2B were much more efficient than liposomes containing GST alone in binding not only AP-2 but also clathrin (Figure 7A). A GST fusion protein comprising the C2A

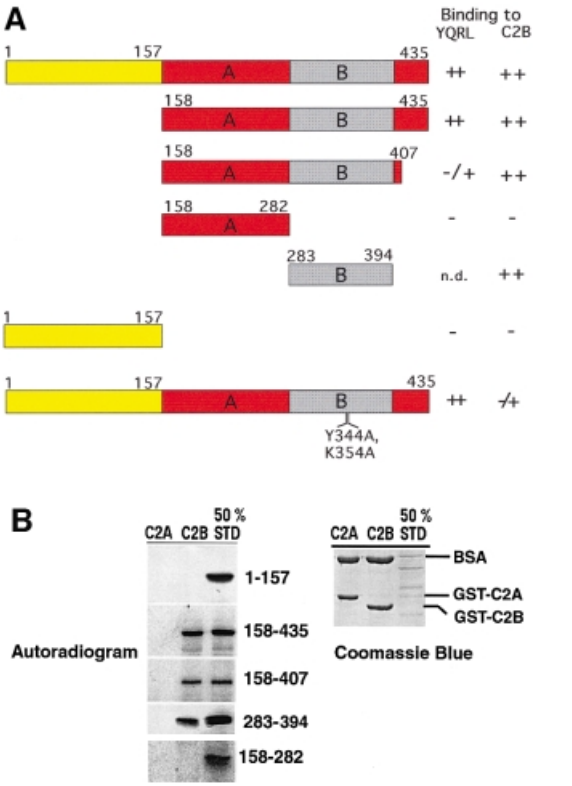

Fig. 3. A synaptotagmin-binding domain within μ2. (**A**) Schematic color representation of full-length μ2 and of μ2 fragments used in this study, and binding of these peptides to either tyrosine-based endocytic motifs (YQRL) or a GST–synaptotagmin C2B (C2B) domain fusion protein. Numbers correspond to amino acids. The A and B subdomains within the crystallized fragment (158–435) of μ2 are shown in red and gray, respectively. (**B**) Fragments of μ2 were synthesized by coupled transcription–translation in the presence of [^{35}S]methionine *in vitro*, incubated with 10 μg/ml GST–synaptotagmin I fusion proteins (C2A or C2B domains; right panel) for 1 h at 4°C and analyzed as described in the legend to Figure 1B. 50% STD = half of the total amount of radiolabeled protein added to the assay.

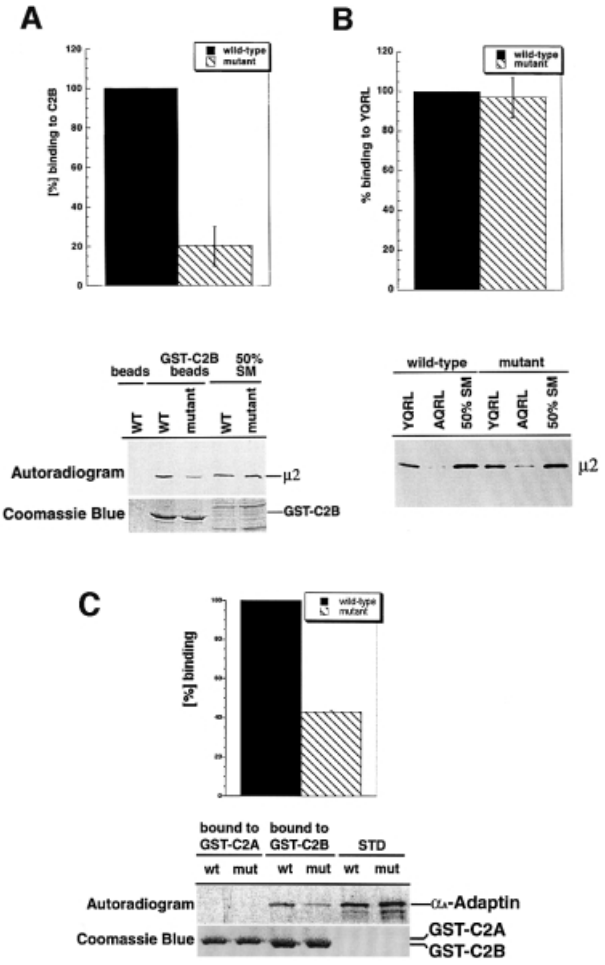

Fig. 4. Adaptor mutants defective for synaptotagmin binding. (**A**) A double point mutation within subdomain B of μ2 reduces the interaction with synaptotagmin. ^{35}S-labeled *in vitro* translated wild-type or mutant (Y344A, K354A) μ2-adaptins were incubated with 10 μg/ml GST–synaptotagmin I fusion proteins (C2A or C2B domains) and analyzed as described in the legend to Figure 1B. 50% SM = half of the total amount of radiolabeled protein added to the assay. Binding of mutant versus wild-type μ2 was quantified from three different, independent experiments and plotted as mean ± SD. (**B**) The mutant form of μ2 retains its ability to recognize tyrosine-based endocytic motifs. ^{35}S-labeled wild-type or mutant (Y344A; K354A) μ2 was incubated with immobilized 14mer peptides containing the C-terminal sequence YQRL or AQRL for 1 h at 4°C in buffer T, and analyzed as described in the legend to Figure 1B. 50% SM = half of the total amount of radiolabeled protein added to the assay.. Binding of mutant versus wild-type μ2 was quantified from three different, independent experiments and plotted as mean ± SD. (**C**) A triple point mutation within α-adaptin reduces the interaction with synaptotagmin. ^{35}S-labeled *in vitro* translated wild-type or mutant (KKK/Q) α-adaptins were incubated with 10 μg/ml GST–synaptotagmin I fusion proteins (C2A or C2B domains) and analyzed as described in the legend to Figure 1B. STD = 1/10 of the total amount of radiolabeled protein added to the assay. Binding of mutant versus wild-type α-adaptin was quantified from three different, independent experiments and plotted as mean ± SD.

domain of synaptotagmin had no effect on coat recruitment (not shown).

Recently, synaptotagmin was shown to cooperate with tyrosine-based endocytic motifs in clathrin/AP-2 recruitment to membranes (Haucke and De Camilli, 1999). More specifically, soluble peptides containing tyrosine-based endocytic motifs were shown to enhance AP-2 binding to the C2B domain of synaptotagmin or to membranes. The liposome model offers the possibility to determine whether such a cooperative interaction can be displayed when both C2B and the tyrosine-based endocytic motifs are membrane bound. In order to test this hypothesis, we prepared liposomes containing either GST or GST–C2B and, in addition, the previously described wild-type (YQRL) or mutant (AQRL) endocytic motif peptides directly conjugated to PE via an additional N-terminal cysteine (Figure 6A). The presence of either the YQRL peptide or the GST–C2B synaptotagmin fusion protein stimulated clathrin/AP-2 recruitment to liposomes compared with control liposomes containing GST and the inactive AQRL peptide. The concomitant presence of both GST–C2B and YQRL peptide produced an even stronger increase in bound clathrin/AP-2. In contrast, neither the YQRL peptide nor GST–C2B stimulated the association of two control proteins, dynamin I and amphiphysin I, with liposomes (Figure 7B). The AQRL peptide or GST had no effect on the binding of either of these proteins (not shown). Similar results were obtained when the coat protein fraction was replaced by rat brain cytosol. The

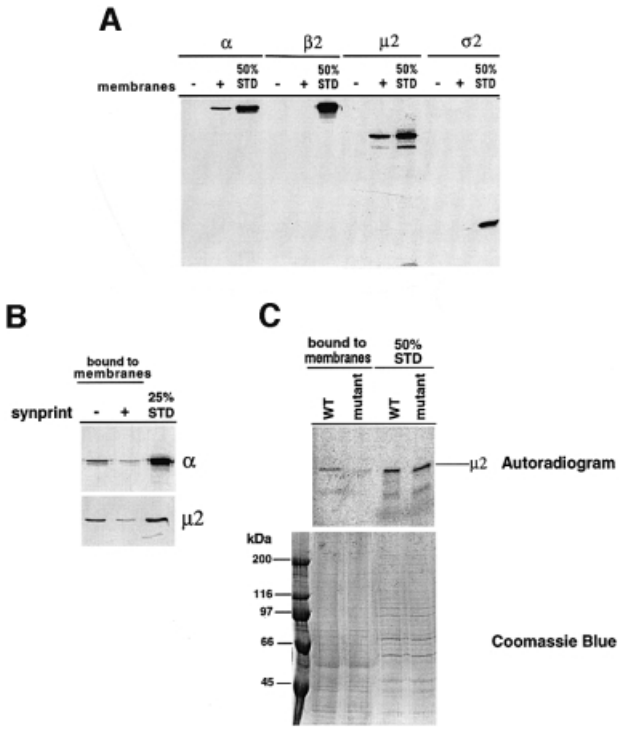

Fig. 5. Synaptotagmin is important for efficient binding of AP-2 subunits to synaptic membranes. (**A**) μ2- and α-adaptin are the major membrane-binding subunits of AP-2. Carbonate-washed LP2 membranes (0.1 mg/ml) were incubated with ^{35}S-labeled *in vitro* translated α-, β2-, μ2- or σ2-adaptins for 10 min at 37°C. Membranes were re-isolated through a sucrose cushion, washed and resuspended in sample buffer. Samples were analyzed by SDS–PAGE and autoradiography. 50% STD = half of the total amount of radiolabeled protein added to the assay. (**B**) Synprint inhibits the recruitment of both μ2- and α-adaptin to synaptic LP2 membranes. Carbonate-washed LP2 membranes (40 μg/ml) were incubated with ^{35}S-labeled *in vitro* translated μ2- or α-adaptin in the presence or absence of 0.3 mg/ml synprint and processed as described in (A). 25% STD = 1/4 of the total amount of radiolabeled protein added to the assay. (**C**) A double point mutation within subdomain B of μ2 reduces the interaction with synaptic LP2 membranes. Carbonate-washed LP2 membranes (40 μg/ml) were incubated with ^{35}S-labeled *in vitro* translated wild-type or mutant (Y344A, K354A) μ2-adaptin and analyzed by SDS–PAGE, staining with Coomassie Blue and autoradiography. 50% STD = half of the total amount of radiolabeled protein added to the assay.

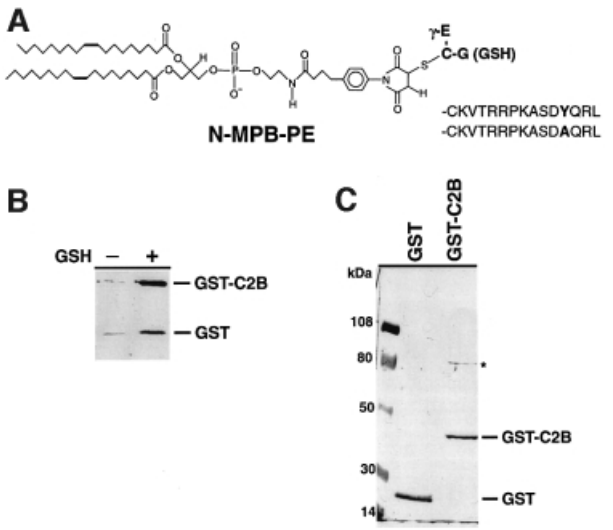

Fig. 6. Liposomes containing glutathione-linked GST fusion proteins or covalently coupled peptides. (**A**) Chemical structure of glutathione (GSH)- or peptide-linked *N*-MPB-PE. (**B**) High affinity binding of GST fusion proteins to liposomes (50 μg of total lipid) containing GSH–PE. A 200 μg/ml aliquot of liposomes containing 20 (w/w) PE (–) or GSH–PE (+) was incubated with 40 μg/ml GST or GST–C2B in PBS for 4 h at 4°C, re-isolated, washed and analyzed by SDS–PAGE and immunoblotting with antibodies against GST. (**C**) Coomassie Blue-stained gel of liposomes (50 μg of total lipid) containing GST or GST–synaptotagmin I C2B domain. Liposomes containing 20% (w/w) GSH–PE were incubated with GST fusion proteins as in (B) and analyzed by SDS–PAGE and staining with Coomassie Blue. The asterisk marks contaminating bacterial DnaK which co-purifies with GST–C2B in small amounts.

presence of GST–C2B on the liposomal surface facilitated clathrin/AP-2 recruitment, but had no effect on the binding of tubulin or dynamin I (Figure 7C).

We also analyzed the recruitment of each of the *in vitro* translated AP-2 subunits to liposomes containing the GST–C2A or GST–C2B fusion proteins of synaptotagmin. Consistent with the results described above, we found that only the α and μ2 subunits exhibited a significant interaction with the GST–C2B-containing liposomes, confirming the role of both subunits in the binding to synaptotagmin (Figure 7D).

Synprint inhibits AP-2 recruitment and endocytosis in fibroblasts

So far, it has remained unclear whether disruption of the interaction between AP-2 and synaptotagmin affects clathrin-mediated endocytosis *in vivo*. We determined whether perturbation of this interaction by the synprint peptide inhibits clathrin-mediated endocytosis in CHO cells. These cells contain a synaptotagmin isoform that interacts with AP-2 (Haucke and De Camilli, 1999). Expression of synprint in CHO cells by cDNA-mediated transfection resulted in an inhibition of the internalization of extracellularly added transferrin (Figure 8A). While transferrin was taken up efficiently by almost all non-transfected cells (95.5 ± 0.7%; mean ± SD), only 15.9 ± 3.1% of the cells expressing synprint were able to internalize transferrin. In view of the specific binding of synprint to the C2B domain of synaptotagmin (see above), this result strongly corroborates the hypothesis that the interaction between synaptotagmin and AP-2 facilitates clathrin-mediated endocytosis in living cells. To determine whether synprint blocks coated pit nucleation, we performed an AP-2 recruitment experiment using bead-attached CHO cell plasma membrane sheets (Haucke and De Camilli, 1999). Upon incubation with rat brain cytosol, AP-2 was being recruited to the plasma membrane and this could be stimulated further by the concomitant presence of a functional tyrosine-based endocytic motif peptide (YQRL) (Figure 8B; see also Haucke and De Camilli, 1999). Addition of synprint inhibited AP-2 recruitment to the membrane in both the presence and absence of YQRL peptide (Figure 8B), suggesting that the endocytic defect seen upon overexpression of synprint was due to impaired adaptor recruitment to the plasma membrane. Membrane binding of tubulin was unaffected by any of these treatments. We conclude that a synaptotagmin isoform participates in receptor-mediated endocytosis in CHO cells by recruiting the clathrin adaptor AP-2 to the plasma membrane.

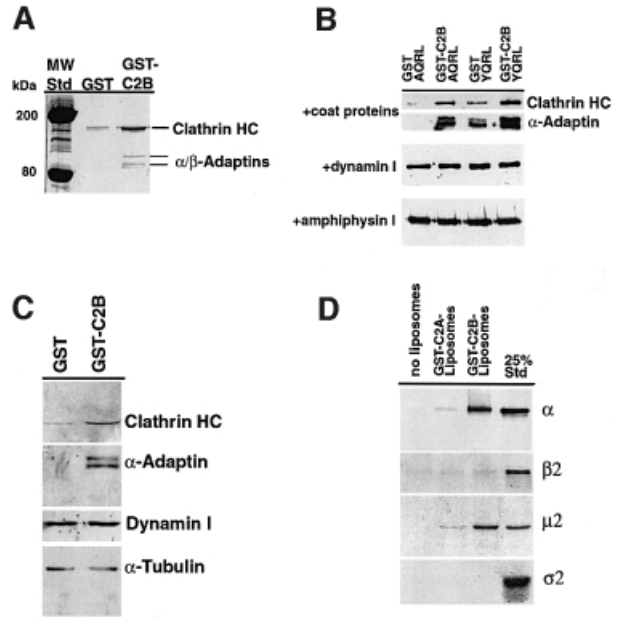

Fig. 7. Synaptotagmin facilitates recruitment of clathrin/AP-2, α-adaptin and μ2 onto liposomes. (**A**) Liposomes (0.5 mg/ml; 50 μg of total lipid) containing glutathione–PE-linked GST or GST–C2B were incubated for 10 min at 37°C with a clathrin coat protein fraction (0.2 mg/ml), re-isolated, washed and analyzed by SDS–PAGE and Coomassie Blue staining. (**B**) Liposomes (0.5 mg/ml; 50 μg of total lipid) containing 20% (w/w) glutathione–PE-linked GST or GST–C2B plus 5% (w/w) YQRL– or AQRL–PE were incubated with either coat proteins (0.2 mg/ml), purified dynamin I (0.1 mg/ml) or amphiphysin I (0.1 mg/ml) (Takei *et al*., 1999) and analyzed as in (B). Equal recovery of the liposomes in all samples was confirmed by measuring the NBD fluorescence of an aliquot of the lipids after re-isolation. (**C**) Liposomes (0.5 mg/ml; 50 μg of total lipid) containing glutathione–PE-linked GST or GST–C2B were incubated for 10 min at 37°C with rat brain cytosol (2.5 mg/ml), re-isolated, washed and analyzed by SDS–PAGE and immunoblotting. (**D**) Liposomes (0.5 mg/ml; 50 μg of total lipid) containing glutathione–PE-linked GST or GST–C2B were incubated with ^{35}S-labeled *in vitro* translated α-, β2-, μ2- or σ2-adaptins for 10 min at 37°C. The liposomes were re-isolated, washed and analyzed by SDS–PAGE and autoradiography.

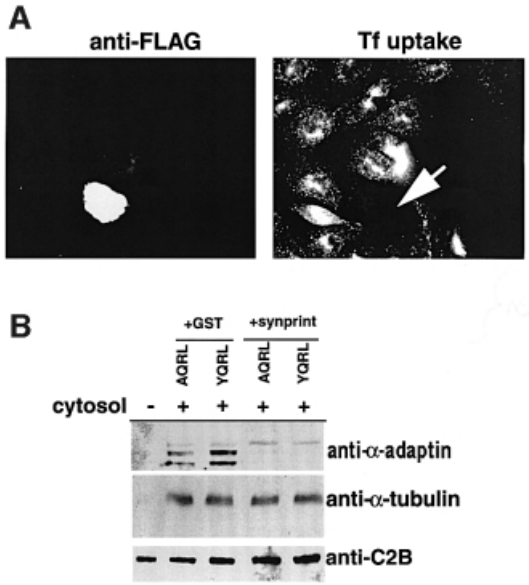

Fig. 8. Disruption of the AP-2–synaptotagmin interaction inhibits clathrin-mediated endocytosis in CHO cells. (**A**) Overexpression of synprint inhibits transferrin (Tf) uptake into CHO cells. FLAG-tagged synprint was transiently expressed in CHO cell fibroblasts. At 24 h post-transfection, cells were incubated with biotinylated transferrin (20 μg/ml) for 10 min at 37°C, washed, fixed and processed for indirect immunofluorescence microscopy using Cy3-conjugated streptavidin and anti-FLAG epitope antibodies. The numbers of endocytosis-competent cells were quantified from three different independent transfection experiments (see text). A total of 606 cells were quantified. (**B**) Synprint inhibits AP-2 recruitment onto CHO cell plasma membrane sheets. Bead-attached inside-out CHO cell plasma membrane fragments were generated as described (Haucke and De Camilli, 1999) and incubated for 15 min at 37°C with rat brain cytosol (4 mg/ml) in the presence or absence of a 200 μM concentration of the indicated tyrosine-based endocytic motif peptides, 0.5 mg/ml synprint (+synprint) or GST (+GST). Samples were washed repeatedly, eluted with sample buffer and analyzed by SDS–PAGE and immunoblotting for α-adaptin, the C2B domain of synaptotagmin and tubulin.

Discussion

In the present study, we have performed a first mapping of the synaptotagmin-binding sites within the AP-2 complex. We have found that the C2B domain of synaptotagmin binds independently to both the α-adaptin and μ2 subunits of AP-2, and primarily to μ2. Multiple interactions of transmembrane proteins with distinct components of vesicle coats have also been reported for COPI-coated vesicles implicated in budding from the Golgi complex (Fiedler *et al*., 1996). Within the μ2 subunit, synaptotagmin binds to a site contained in its subdomain B that is distinct from the tyrosine motif-binding site. The dual contact of synaptotagmin with both μ2- and α-adaptin may explain why tyrosine-based endocytic motifs stimulate the interaction of synaptotagmin with AP-2, but not with μ2 alone. Most probably, occupancy of the tyrosine-based motif-binding site generates a conformational change within AP-2 that modifies its complex interacting surface with synaptotagmin. Interactions between α-adaptin and μ2 subunits of AP-2 and transmembrane cargo proteins of clathrin-coated vesicles have been reported previously (Nesterov *et al*., 1995; Kirchhausen *et al*., 1997; Bonifacino and Dell'Angelica, 1999; Fernandez-Chacon *et al*., 2000). Synaptotagmin, however, is the only known protein so far that binds both subunits. This property supports the hypothesis that members of the synaptotagmin family play a key role in the recruitment of AP-2 to sites of clathrin coat assembly. Synaptotagmin isoforms are present in all cells, and at all sites where AP-2/clathrin coats were shown to assemble, including lysosomes (Li *et al*., 1995; Butz *et al*., 1999; Haucke and De Camilli, 1999; Martinez *et al*., 2000).

Our data provide new evidence for the hypothesis that μ2 and the core region of α-adaptin represent major interfaces of AP-2 with the membrane (Nesterov *et al*., 1995; Ohno *et al*., 1995; Gaidarov and Keen, 1999). Multiple interactions of these subunits with membrane proteins may enhance membrane attachment either by additivity or by acting synergistically to increase the affinity of individual binding sites. Furthermore, they may represent a mechanism to recruit a multiplicity of cargo molecules into coated pits. In the case of synaptic vesicles, this multiplicity of interactions could be one of the mechanisms through which internalization of different synaptic vesicle proteins is coordinated. Phosphoinositides, including phosphatidylinositol (4,5)-bisphos-

phate (Cremona *et al*., 1999; Gaidarov and Keen, 1999) and phosphoinositides containing a phosphate at the 3′ position (Rapoport *et al*., 1997) presumably also synergize with coat–membrane protein interactions. Other factors, such as other lipid changes or focal changes in the submembranous actin cytoskeleton, may also contribute to clathrin-coated pit formation either directly or by providing a restrictive meshwork that delineates so-called 'hot-spots' of endocytosis (Roos and Kelly, 1999).

Besides the direct binding of synaptotagmin to AP-2 subunits, other results of this study demonstrate that these interactions are critical for the recruitment of clathrin/AP-2 to either synaptic or fibroblastic plasma membranes and may thus facilitate clathrin-coated pit nucleation *in vivo*. First, the synaptotagmin-binding peptide synprint (Chapman *et al*., 1998; this study) inhibits membrane recruitment of α-adaptin and μ2 as well as clathrin-mediated endocytosis *in vivo*. Secondly, a point mutation within the synaptotagmin-binding region of μ2 compromises the ability of the protein to bind to either synaptotagmin or to synaptic membranes. Thirdly, and most importantly, the presence of a synaptotagmin C2B domain fusion protein on liposomal membranes devoid of other membrane proteins stimulates clathrin/AP-2 recruitment. This recruitment is increased further by the presence of tyrosine-based endocytic motifs at the membrane interface, as previously demonstrated for the recruitment of AP-2 to native membranes (Haucke and De Camilli, 1999). Consistent with these findings and with the idea that membrane proteins can act as primers for vesicle budding (Springer *et al*., 1999), it has been found that the cytoplasmic tails of p24 family proteins can facilitate the formation of COPI-coated vesicles on liposomal membranes (Bremser *et al*., 1999).

In conclusion, our data strongly support a key role for synaptotagmin in clathrin coat recruitment, in agreement with recent findings in stably transfected HeLa cells (von Poser *et al*., 2000). At least in nerve terminals, where the formation of clathrin-coated pits is crucially dependent upon the previous incorporation of synaptic vesicle proteins into the plasma membrane (Heuser and Reese, 1973; Gad *et al*., 1998), synaptotagmin may serve as a trigger for coat assembly. Since clathrin coats do not assemble on synaptic vesicles prior to exocytosis, it will be of interest to understand how this endocytic function of synaptotagmin is masked until exocytosis has occurred. Possible mechanisms may include conformational changes in its cytoplasmic domain, dissociation of binding proteins or a synergistic action of synaptotagmin with specific membrane lipids that are generated only after exocytosis.

Materials and methods

Materials

Crude brain lipid extracts (type I Folch fraction I) were from Sigma, St Louis, MO; *N*-MPB-PE, PE and NBD-labeled PE were from Avanti Polar Lipids. Plasmids encoding wild-type α_A-adaptin or a triple lysine (KKK/Q) mutant thereof were kindly provided by Dr Jim Keen, and plasmids encoding AP-2 subunits were provided by Dr J.S.Bonifacino. Antibodies against the C2B domain of synaptotagmin and β1/β2-adaptins were gifts of Drs Tom Martin and Tom Kirchhausen, respectively. All other reagents used have been described before (Haucke and De Camilli, 1999).

Molecular biology procedures

Constructs encoding full-length α-, β2-, μ2- and σ2-adaptins or fragments of μ2-adaptin were generated by PCR using plasmid DNA as a template (a kind gift of Dr J.S.Bonifacino, NIH), subcloned into pcDNA3 and verified by restriction analysis and DNA sequencing. A His_6-tagged mutant of μ2-adaptin harboring mutations Y344A/K354A was made by PCR, cloned into pcDNA3 and verified by DNA sequencing. ^{35}S-radiolabeled proteins were synthesized *in vitro* by coupled transcription–translation using reticulocyte lysate according to the manufacterer's instructions (Promega). Post-ribosomal supernatants were then generated by centrifugation at 200 000 *g*. Standard techniques were used for preparation of plasmid and genomic DNA, restriction analysis, PCR and cloning of DNA fragments.

Lipopeptide synthesis and generation of liposomes

PE was conjugated to either glutathione or wild-type and mutant 15mer peptides (referred to as YQRL or AQRL, respectively) according to Bremser *et al*. (1999) using *N*-MPB-PE (Avanti Polar Lipids) as lipid acceptor. High coupling efficiency was verified by measuring the peptide content of the modified *N*-MPB-PE after extraction using free reduced glutathione or soluble peptides as a standard. Liposomes were made as described in Takei *et al*. (1998) using a bovine brain crude lipid extract (type I Folch fraction I; Sigma, St Louis, MO), supplemented with 0.05% (w/w) NBD-labeled PE and 20% (w/w) PE or glutathione–PE, or an additional 5% (w/w) YQRL– or AQRL–PE for the experiment shown in Figure 7C. Liposome recovery was followed by measuring NBD fluorescence (λ_{EX} = 460 nm; λ_{EM} = 534 nm) on a Hitachi fluorimeter after re-isolation of the liposomes.

Protein recruitment to membranes and liposomes

For biochemical analysis of protein recruitment onto synaptic membranes (LP2 fraction) or CHO cell plasma membranes, published procedures were used (Haucke and De Camilli, 1999). Proteins incubated with liposomes were loaded on a bed of 1 vol. of cytosolic buffer (Takei *et al*., 1998) on top of 1 vol. of 1.0 M sucrose in cytosolic buffer and centrifuged at 150 000 *g* for 30 min at 4°C. Liposomes were collected from the interface, washed with 200 μl of cytosolic buffer, resuspended at 10 mg/ml in cytosolic buffer, solubilized in sample buffer and analyzed by SDS–PAGE.

Miscellaneous

Rat brain cytosol was prepared according to Takei *et al*. (1998). GST fusion proteins, clathrin coat proteins, LP2 synaptic membranes and CHO cell plasma membrane lawns on beads were prepared as described previously (Haucke and De Camilli, 1999). His_6-tagged synprint was purified as in Chapman *et al*. (1998). Standard procedures were used for indirect immunofluorescence, SDS–PAGE and immunoblotting.

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