Recognition of a basic AP-2 binding motif within the C2B domain of synaptotagmin is dependent on multimerization

Isabelle GrassŠ, Stefanie Thiel*Š, Stefan Höning* and Volker Haucke*†

†Institut für Chemie-Biochemie, Freie Universität Berlin, Takustraße 6, D-14195 Berlin, Germany;
*Zentrum für Biochemie & Molekulare Zellbiologie, Department of Biochemistry II, University of Göttingen, Humboldtallee 23, D-37073 Göttingen, Germany

†To whom correspondence should be addressed:
phone: +49-30-838 56922
fax: +49-30-838 56919
e-mail: vhaucke@chemie.fu-berlin.de

Šthese authors contributed equally to this study

Running title: Recognition of basic endocytosis signals by AP-2µ
Summary

Synaptotagmin is a multifunctional membrane protein which may regulate exo-endocytic cycling of synaptic vesicles at the presynaptic plasmalemma. Its C2B domain has been postulated to interact with a variety of effector molecules including acidic phospholipids, phosphoinositides, SNAREs, calcium channels, and the clathrin adaptor complex AP-2. Here we report that a basic motif within the C2B domain is required and sufficient for binding to AP-2 via its µ2 subunit and that this interaction is dependent on multimerization of the AP-2 binding site. Moreover, we show that upon fusion to a plasma membrane reporter protein this sequence is sufficient to target the chimeric molecule for internalization. We hypothesize that basic motifs within multimeric membrane proteins may represent a novel type of clathrin/ AP-2-dependent endocytosis signal.
**Introduction**

Clathrin-mediated endocytosis plays an important role in the recycling of at least a subpopulation of synaptic vesicles (SVs) at the presynaptic plasmalemma. This process involves the regulated and directed assembly of clathrin, AP180, epsin, and AP-2 adaptor complexes along with several accessory proteins at endocytic 'hot spots' surrounding the active zone (1-4).

Genetic and biochemical evidence has implicated the multimeric SV membrane protein synaptotagmin (5) in the regulation of both exo- and endocytic cycling of presynaptic vesicles (6,7). Among the conserved properties of different members of the synaptotagmin family is their ability to form disulfide-crosslinked oligomers (6,8-10). Via their C2 domains synaptotagmins can associate with calcium and acidic phospholipids (11,12). Biochemical studies using recombinant proteins have shown that the second C2 domain (C2B) of most, if not all synaptotagmins harbors a binding site for the clathrin adaptor complex AP-2 (13) which at least partially overlaps with the binding site for N-type calcium channels (14). Moreover, based on coimmunoprecipitation (15), two-hybrid analysis (16), and affinity chromatography the synaptotagmin binding site within AP-2 has been mapped to the carboxy-terminal portion of its µ2 subunit (17).

Consistent with an endocytic function of synaptotagmin at the synapse it has been demonstrated that worms or flies expressing a truncation mutant of synaptotagmin I that lacks its C2B domain display reduced numbers of SVs (18,19). Acute inactivation of tagged transgenically expressed synaptotagmin I in nullstrains results in severe vesicle recycling defects (20), presumably owed to defective clathrin-mediated synaptic vesicle endocytosis. Microinjection of antibodies directed against the AP-2 binding cytoplasmic domain of synaptotagmin I into the giant synapses of squid (21) or lamprey also result in severe vesicle depletion and accumulation of endocytic vacuoles (22). Lastly, overexpression of truncated synaptotagmin lacking its C2B domain exerts a dominant-negative effect on endocytosis and clathrin-coated pit assembly and this is strictly dependent on cysteine-mediated multimerization (8).
We report here that a basic motif within the synaptotagmin C2B domain is required and sufficient for binding to AP-2 via its \( \mu_2 \) subunit and that this interaction is dependent on multimerization of the AP-2 binding site.

**Experimental Procedures**

*Recombinant proteins.*

GST- or histidine-tagged recombinant proteins were purified according to the manufacturer's instructions from triton X-100-solubilized bacterial homogenates. In order to obtain nucleic acid-free monomeric GST fusion proteins recombinant bead-bound proteins were treated with 1 mg/ml RNaseA plus 1mg/ml DNase I in HBS (pH 7.4) containing 1 mM PMSF. Samples were washed twice with 10 volumes of high-salt HBS (HBS containing 1 M NaCl) and finally suspended in standard HBS (pH 7.4). This procedure effectively removed essentially all \( A_{260} \)-absorbing nucleic acid contaminants as determined by measuring the \( A_{280} / A_{260} \) ratio.

Recombinant AP-2 'cores' were purified as described in (23).

*Peptides.*

Peptides were synthesized by standard solid-phase synthesis, HPLC-purified and verified by mass spectrometry. Peptide sequences were as follows. Synaptotagmin I-derived KR-peptide: (C)KRLKKKKTTIKK; TGN38-derived tyrosine motif peptide: (C)KVTRRPASDYQRL; CD3\( \gamma \)-derived di-leucine motif peptide: RQSRASDKQTLPLN.

*Preparation of liposomes and liposome binding assay.*

Unilamellar liposomes were prepared as described (24). Briefly, dried lipids (60% PC, 20% PE plus 20% (w/w) of either PC or PS) were rehydrated in 0.3M sucrose, reisolated and resuspended in 20 mM HEPES-KOH (pH7.4). Binding assays were carried out by incubating 2 \( \mu \)g purified \( \mu_2 \) (aa 157-435) with 100 \( \mu \)g liposomes in cytosolic buffer (24) in the presence or absence of calcium or EGTA.
for 15 min at RT. Liposomes were reisolated by ultracentrifugation, washed and bound proteins were analyzed by SDS-PAGE.

*Liposome incubation of washed GST-C2B.*

Washed GST-C2B (2 mg/ml; in 20 mM HEPES-KOH pH 7.4) was preincubated with 2 mg/ml liposomes (60% PC, 20% PE plus 20% of either PC or PS (w/w)) plus 1 mM CaCl2 for 30' at RT, then for 45' at 4°C (rotating wheel). Samples were washed once in 20 mM HEPES-KOH pH 7.4. Liposomes were disrupted by addition of 1% Triton X-100 in HBS (pH 7.4) and binding of AP-2 (from coat proteins) or µ2 was analyzed as described below.

*Oxidation/ reduction of cysteine-linked fusion proteins.*

To obtain mono- or dimeric GST-Cys-KR (or Cys-KR) or multimeric GST-Cys6-C2B fusion proteins the high-salt-treated proteins bound to beads were incubated with 1 mM DTT or 200 µM copper(II)-sulfate plus 600 µM (1,10)-phenanthroline for 30 min at room temperature. Samples were washed three times with HEPES-buffered saline and resuspended in HBS. Proteins were used within 24 h after purification.

*Pulldown experiments.*

His6-tagged µ2 (50 µg) immobilized on beads was incubated with CHAPS-extracted rat brain synaptosomes (1 mg/ml) in buffer A (10 mM HEPES-KOH pH 7.4, 100 mM NaCl, 1% CHAPS, 1mM PMSF plus protease inhibitor cocktail) for 2 h at 4°C. Samples were washed 3X with 50 volumes of buffer A, eluted with SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting. For studies using recombinant GST, GST-KR or GST-(Cys)-C2B clathrin coat proteins (60 µg/ml) or recombinant µ2 (30 µg/ml) were diluted into HEPES-buffered saline containing 1 mM PMSF plus 1% triton X-100 and incubated with 30 µg of the respective GST fusion protein in a final volume of 0.5 mL for 2 h at 4°C. Samples were washed thoroughly, eluted
with SDS-PAGE sample buffer and analyzed by SDS-PAGE and staining with Coomassie Blue or immunoblotting.

**Immunoprecipitations.**

Rat brain synaptosomes were kept in 20 mM HEPES-KOH (pH 7.4)/320 mM sucrose/50 mM KCl/1 mM CaCl$_2$. Solubilization buffer was added to give a final concentration of 20 mM HEPES-KOH (pH 7.4)/160 mM sucrose/100 mM KCl/400 μM CaCl$_2$/2 mM MgCl$_2$/1% triton X-100 at a protein concentration of 3 mg/ml. Solubilized material was precleared by centrifugation, synthetic peptides (100 μM) were added where indicated, and finally samples were subjected to immunoprecipitation with monoclonal antibodies against γ-glutamic acid decarboxylase (GAD6; control) or α−adaptin (clone AP.6) for 4 h at 4°C. Beads were washed 4X and bound proteins were eluted with SDS-PAGE sample buffer.

**Transfection and internalization assay.**

PC12 or HEK293 cells were transiently transfected with plasmids encoding c-myc-synaptotagmin I, CD4, tetrameric CD4$^{tailless}$-AA, or tetrameric CD4$^{tailless}$-KR using Lipofectamine 2000™ and assayed 24 h post-transfection. Cells were starved in serum-free medium for 2 h. PC12 cells were chilled on ice, then anti-c-myc were allowed to bind for 1 h at 4°C. For HEK293 cells anti-CD4 antibodies (Q4120) were diluted in serum-free medium, added directly to the cells, and allowed to become internalized. Cells were incubated for 30 min at 37°C, thoroughly rinsed with PBS, fixed with 4% paraformaldehyde, and decorated with Alexa$^{488}$-labelled secondary antibodies under non-permeabilizing conditions for 2 h at RT. Remaining surface-exposed antibodies were blocked by adding excess unlabeled goat-anti-mouse IgG. Internalized protein was detected by decoration with Alexa$^{594}$-labelled secondary antibodies after triton X-100 permeabilization. For quantification CD4 chimera expressing cells were scored on a relative scale. Cells undergoing 'strong internalization', had internalized the majority of CD4 or CD4 chimeras during the 30 min incubation period. Transfected cells were scored as undergoing 'weak-to-moderate' internalization, if a minor fraction
of the expressed protein had been endocytosed. For quantification of myc-synaptotagmin I endocytosis a binary mask was applied to determine the ratio of internalized over surface-exposed myc-synaptotagmin I from epifluorescent images using SlideBook software (3i Inc., USA).

Results

Recent data from several laboratories have implicated basic sorting signals in the regulation of ligand-dependent internalization of multimeric membrane proteins such as AMPA-type glutamate and \( \alpha \)1B-adrenergic receptors via association with the \( \mu \)2–subunit of the clathrin adaptor complex AP-2 (25,26). A similar basic motif has been shown to function as the major AP-2 binding site within the C2B domain of the multimeric SV membrane protein synaptotagmin (5,14). Mutation of this basic motif has been shown to block the direct association of GST-tagged recombinant synaptotagmin I with \( \mu \)2–adaptin (17), suggesting that it may connect synaptotagmin to the endocytosis machinery. In order to confirm this hypothesis we investigated the ability of mutated synaptotagmin I in which two critical lysine residues within the presumed AP-2 binding site (Fig. 2A, asterisk) have been mutated to alanines to undergo endocytosis in PC12 cells. In agreement with data from other laboratories (27) we found that mutant synaptotagmin I was compromised in its ability to become internalized from the cell surface (Fig.1A, B). This indicates that two conserved lysine residues within the basic AP-2 binding site of synaptotagmin I-C2B are required for efficient endocytosis in PC12 cells.

Association of native synaptotagmin I with AP-2 or recombinant \( \mu \)2 can be blocked by a basic C2B domain-derived peptide

Basic lysine-rich sequences comprising the putative AP-2 binding site within the C2B domain are highly conserved between different synaptotagmin family members in mammals (Fig. 2A). In order to find out whether this lysine-rich sequence within C2B contributes to the association of native synaptotagmin I with endogenous AP-2 in the brain we performed co-immunoprecipitation
experiments in the presence or absence of a synthetic peptide comprising the putative AP-2 binding site of synaptotagmin I (KR peptide). We immunoprecipitated AP-2 and associated proteins from triton-X-100-lysed rat brain synaptosomes in the presence or absence of the basic peptide and analyzed the samples for the presence of synaptotagmin I and clathrin. Consistent with previous mapping studies (14) synaptotagmin I could be detected in mock-treated samples but was absent from immunoprecipitates obtained from KR peptide-treated lysates. By contrast, the association of clathrin with AP-2 remained unaffected (Fig. 2B). To test whether the association of AP-2 and native synaptotagmin I involved µ2 we performed affinity chromatography experiments. As expected hexahistidine-tagged µ2-adaptin selectively pulled down synaptotagmin I but not synaptophysin, another major synaptic vesicle protein (Fig. 2C) and this association was blocked by addition of the basic synaptotagmin-derived (KR) peptide (Fig. 2D). No effect on binding of synaptotagmin I to µ2 was seen if a di-leucine motif containing peptide was taken instead. These data indicate that native synaptotagmin I binds to AP-2 via interactions of µ2 adaptin with a basic motif within the C2B domain.

**Binding of synaptotagmin-C2B to µ2-adaptin in vitro is modulated by oligomerization**

Since many of the proteins carrying basic internalization motifs are multimeric membrane proteins we wondered whether binding of synaptotagmin's C2B domain to AP-2µ might be modulated by oligomerization. To this aim we studied the interaction between synaptotagmin I and µ2 further by affinity chromatography using recombinant bacterially expressed fusion proteins. Conventionally purified GST-C2B displayed robust binding to µ2-adaptin (Fig. 3A, untreated). C2 domains have been shown to form non-covalent oligomers (6,28). Moreover, bacterially expressed recombinant C2B has been shown to contain nucleic acid contaminants which may facilitate oligomerization and/or affect its interaction with other molecules. We therefore analyzed the ability of high-salt washed, nuclease-treated monomeric GST-C2B to associate with µ2-adaptin. Surprisingly, this treatment rendered C2B almost incapable of binding to µ2 (Fig. 3A, washed). The binding of negatively charged nucleic acids to recombinant C2B may reflect the propensity of the
molecule to associate with negatively charged phospholipid headgroups which in the case of phosphatidylserine (PS) has been shown to induce calcium-triggered C2 domain oligomerization (12). Calcium-induced synaptotagmin oligomerization is likely to be of physiological relevance during excitation-secretion coupling. Hence, we analyzed whether addition of PS and calcium would restore the ability of GST-C2B to bind to AP-2μ. Indeed, pretreatment of washed GST-C2B with PS-containing liposomes in the presence of calcium completely restored its ability to bind to purified μ2. Other lipids such as phosphatidylcholine (PC; Fig.3A), phosphatidylaethanolamine, phosphoinositides including PI(4)P, PI(4,5)P₂, PI(3,4,5)P₃ or inositolpolyphosphates (supplementary figure 1) were ineffective. No binding of μ2 to washed GST-C2B was seen in the presence of EGTA. This effect was unlikely due to a bridging function of μ2, since μ2 itself was unable to bind to PS-containing liposomes irrespective of the presence of calcium, but was instead capable of associating with PIP₂ (Fig. 3B) which did not restore μ2-binding to C2B. Similar results were seen for the association of washed GST-C2B with native AP-2 from brain extracts (Fig. 3C). Pretreatment of bead-bound GST with PS-containing liposomes did not result in AP-2 binding (data not shown). The interaction of PS-treated, washed GST-C2B with AP-2 displayed characteristics previously described for the interaction of native synaptotagmin I with AP-2 adaptors (15). Addition of a tyrosine-based endocytic sorting motif peptide (YQRL) stimulated the association of PS-treated GST-C2B with AP-2 from brain whereas an inactive mutant (AQRL) had no effect (supplementary figure 2). We also analyzed the binding of recombinant GST-C2B to synprint, the synaptotagmin binding site of N-type calcium channels (29). As seen for AP-2, high-salt treatment of recombinant GST-C2B rendered the fusion protein unable to recognize histidine-tagged synprint. However, binding activity could be restored by preincubation with PS- but not PC-containing liposomes (Fig. 3D). Thus, negatively charged PS can restore the ability of nucleic acid-free C2B to associate with either AP-2 or N-type calcium channels.

**Binding of recombinant synaptotagmin I C2B domain to native AP-2 or recombinant μ2-adaptin is dependent on multimerization**
To test directly whether the ability of C2B to bind to AP-2 is dependent on synaptotagmin oligomerization, we constructed chimeric fusion proteins comprising GST, a tetracysteine-containing peptide linker, and the C2B domain of synaptotagmin I. The oligomerization state of such a chimeric protein can be controlled in vitro by adjusting the redox conditions (30). In the presence of the reducing agent DTT, the fusion protein migrated as a single band of approximately 42 kDa. Copper (II)-phenanthroline-catalyzed oxidation of the cysteine residues in the spacer peptide resulted in the formation of disulfide-linked oligomers, mostly trimers and tetramers of 85 and 140 kDa, respectively (Fig. 4A). We then assayed mono- or multimeric, washed GST-Cys-C2B fusion proteins for their ability to bind to µ2-adaptin. Strikingly, only oxidized, multimeric forms of the C2B fusion protein bound effectively to µ2, whereas reduced, monomeric C2B did not bind in the absence of PS and calcium (Fig. 4B). Similar results were seen for native AP-2 complexes (Fig. 4C). No binding to GST pretreated with oxidizing or reducing reagents could be detected. The association of multimeric GST-Cys-C2B with AP-2 was specific, as it could be inhibited by low micromolar concentrations of the basic C2B-derived (KR) but not by a dileucine motif-containing peptide (Fig. 4D). These data indicate that multimerization might be a prerequisite for the recognition of the basic AP-2 binding motif within the C2B domain of synaptotagmin I.

The basic C2B peptide is sufficient for binding to AP-2 or µ2-adaptin

To determine whether the basic motif within the C2B domain is not only required but also sufficient for AP-2 binding, we created a GST-fused synaptotagmin peptide (GST-KR). Upon incubation with triton X-100 extracted rat brain homogenate the GST-KR peptide pulled down AP-2 and this interaction could be competed by addition of the chemically synthesized soluble KR peptide. No competition was seen if a di-leucine-motif-containing control peptide was added instead. Concomitant presence of a peptide encompassing a tyrosine-based endocytosis motif (YQRL) stimulated the interaction between native AP-2 and the GST-KR fusion protein (Fig. 5A). GST-KR was also capable of efficiently interacting with purified recombinant µ2 and this interaction could be blocked by addition of soluble KR but not by the YQRL peptide (Fig. 5B). In
This case, no stimulatory effect of the YQRL peptide could be detected, in agreement with earlier findings using GST-C2B and \textit{in vitro} translated \(\mu2\) (17).

Since recognition of the C2B domain apparently is dependent on multimerization we set out to define the minimal requirements for this association using the GST-KR peptide chimera and recombinant AP-2 core complexes (23). To this aim we constructed a fusion protein in which the basic KR peptide was connected to GST via a single cysteine residue. Upon exposure to oxidizing conditions this fusion peptide dimerizes. As seen before for the intact C2B domain, high-salt-washed reduced GST-Cys-KR was incapable of binding to recombinant AP-2 cores, but binding activity could be restored by cystine-mediated dimerization (Fig. 5C). Fully assembled AP-2 core complexes lacking the distal 278 amino acids of \(\mu2\) encompassing the putative synaptotagmin binding site (17) were unable to bind to GST-Cys-C2B (Fig. 6A) or GST-Cys-KR (not shown) irrespective of its oligomerization status. We then measured the affinity of chemically synthesized, mono- or dimeric KR peptides (more than 95% pure) for AP-2 cores by surface plasmon resonance (SPR). Dimerization drastically increased the affinity of the C2B-derived KR peptide for AP-2 cores about 50-fold, resulting in high-affinity binding with a dissociation constant of about 220 nM (Fig. 6B).

The basic KR peptide supports endocytosis of tetrameric CD4 chimeras in living cells

We finally wanted to determine the physiological relevance of the interaction between the basic KR motif derived from synaptotagmin I and AP-2 for the internalization of plasma membrane receptors in living cells. To assess the ability of the KR peptide to facilitate internalization of an otherwise non-endocytosed reporter protein we grafted the basic motif onto the extracellular and membrane-spanning domains of the T cell surface glycoprotein CD4 (31). We transfected HEK293 cells with constructs encoding either wild-type CD4 which bears a di-leucine-type internalization motif within its cytoplasmic domain, or artificially tetramerized (32) tailless mutants in which the cytoplasmic tail of CD4 had been replaced with the basic C2B domain peptide from synaptotagmin I (CD4\textsubscript{tailless}-KR), or a poly-alanine control peptide (CD4\textsubscript{tailless}-AA). Wild-type CD4 as well as
tetrameric CD4tailless-KR were robustly internalized whereas the tetrameric CD4tailless-AA mutant was not (Fig. 7A, B). Weak internalization was detectable if the C2B domain peptide was presented in the context of monomeric CD4 (not shown). These data indicate that presentation of the basic synaptotagmin-derived AP-2 binding peptide as a tetramer is sufficient to restore internalization of a tailless CD4-chimeric mutant protein.

**Discussion**

In the present study we have investigated the interaction between the cycling synaptic vesicle membrane protein synaptotagmin and the endocytic clathrin adaptor complex AP-2. Consistent with earlier mapping studies (14,17) we have found that a basic region within the C2B domain of synaptotagmin I is required and sufficient for the association with AP-2 which mainly occurs via its µ2 subunit. Based on *in vitro* experiments using recombinant fusion proteins and functional studies in living cells we have revealed a requirement for synaptotagmin-C2B multimerization for recognition by the µ2 subunit of AP-2 or binding to the synprint region of N-type calcium channels (29). This interaction is specific as it could be inhibited by a basic peptide encompassing the putative AP-2 and synprint binding site within the C2B domain (14). Moreover, we have demonstrated here that a chemically synthesized dimeric KR peptide binds to AP-2 with nanomolar affinity. Similarly, mutations within this basic stretch have been shown to abolish the interaction between synaptotagmin I and AP-2 or synprint both *in vitro* (14) and in transfected neuroendocrine cells (27). Multimerization of the C2B domain or the basic motif can be accomplished by two distinct mechanisms *in vitro*: calcium-dependent binding to acidic phospholipids (12) or cysteine-mediated crosslinking. Indeed, both mechanisms have been described to mediate calcium-dependent as well as calcium-independent homo- and heterooligomerization of native synaptotagmins (5,6,10,28). We thus believe that our results are of physiological relevance. Multimerization may therefore be a prerequisite for the correct functioning of synaptotagmin in excitation-secretion coupling via calcium channels as well as in SV membrane retrieval.
The finding that synaptotagmin binding to AP-2 may depend on the correct assembly of synaptotagmin into multimers lends further support to a number of biochemical (13,15), genetic (18-20) and physiological studies (20-22) that suggest a role for synaptotagmin in SV recycling, in addition to its established function in calcium-dependent neurotransmitter release (6,7). Synaptotagmin internalization in vivo in addition to AP-2 presumably depends on or is facilitated by other adaptor proteins such as stonin 2, a synaptotagmin I- and AP-2 binding protein in brain (33,34).

Basic endocytosis signals that are recognized by AP-2 complexes and perhaps other adaptors have recently been identified in a variety of membrane proteins. These include the GluR1-3 subunits of ionotropic AMPA-type glutamate receptors at excitatory synapses (25), the \(\alpha_{1B}\)-adrenergic receptor (AR) (26), Na,1.2 sodium channels (35), and the cycling ER-Golgi-intermediate compartment protein 53 (ERGIC-53) (36). A similar motif is also present in the transcytotic polymeric immunoglobulin receptor (see (25)) and in the \(\gamma2\) and \(\beta3\) subunits of inhibitory GABA\(_\Lambda\) receptors. A common feature of most of these proteins is their ability to become internalized in response to activation or ligand binding. In the case of the AMPA receptor subunit GluR2 it has been demonstrated that the basic AP-2 binding site within its cytoplasmic tail is required for NMDA-induced AMPA receptor endocytosis and synaptic long-term depression (LTD) (25). Preliminary experiments indicate that GluR2 and synaptotagmin I in fact use a common \(\mu2\)-dependent mechanism of association with AP-2. Similarly, agonist-induced activation of a basic motif within the \(\alpha_{1B}\)-adrenergic receptor facilitates its association with AP-2\(\mu\) and concomitant receptor internalization (26). Strikingly, all proteins displaying basic internalization motifs including synaptotagmin I, ERGIC-53, pIgR, AMPA\(\Lambda\), GABA\(_{\Lambda\gamma}\), and \(\alpha_{1B}\)-adrenergic receptors are oligomers (5,35-37). It thus appears that the basic sorting motif within the C2B domain of synaptotagmin I may be part of a family of related signals that facilitate regulated internalization of multimeric membrane proteins. Multimerization-dependent interactions of membrane proteins with sorting adaptors have also been implicated in regulating surface delivery of octameric \(K_{ATP}\) channels via an arginine-based signal (32).
Footnotes

¶ Kastning et al., manuscript in preparation
¶¶ Kittler et al., manuscript in preparation

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References

Legends to figures

Figure 1: Internalization of synaptotagmin I requires a lysine-rich sequence within its C2B domain.

Internalization of wild-type or mutant (K₃₂₆,K₃₂₇AA) c-myc epitope-tagged synaptotagmin I from the surface of transfected PC12 cells. Endocytosis (30' @ 37°C) was monitored by an antibody feeding assay using anti-myc antibodies (9E11).

(A) Representative confocal images of internalized (labeled with Alexa⁵⁹⁴-red) and surface exposed (labeled with Alexa⁴⁸⁸-green) synaptotagmin I. Merged images are shown. Scale bar, 10 µm.

(B) Data shown in (A) were quantified as described in the experimental procedures. Internalization is depicted as the ratio of Alexa⁵⁹⁴ (internalized)- to Alexa⁴⁸⁸ (surface-exposed)-labeled synaptotagmin I wild-type or KKAA mutant.

Figure 2: Interaction of native synaptotagmin I with AP-2 or µ2-adaptin can be blocked by a C2B domain-derived peptide

(A) Multiple sequence alignment of lysine-rich, putative AP-2 binding sequences from the C2B domains of rat synaptotagmins I-XIII using the Clustal W method. The conserved basic cluster is boxed in red, lysines 326 and 327 determined to be critical for synaptotagmin I internalization are indicated by an asterisk.

(B) Co-immunoprecipitation of synaptotagmin I with AP-2 from synaptosomal extracts. CHAPS-extracted rat brain synaptosomes were incubated with 100 µM of basic KR peptide (see experimental procedures for details) or mock-treated and subjected to immunoprecipitation with antibodies against AP-2α or γ-glutamic acid decarboxylase (GAD6; Ctrl). The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting against AP-2α, clathrin heavy chain (HC), and synaptotagmin I (syt I).

(C) Affinity purification of synaptotagmin I from CHAPS-extracted rat brain synaptosomes (1 mg/ml) using His₆-tagged (50 µg) analyzed by SDS-PAGE and immunoblotting for clathrin heavy chain
(HC), synaptotagmin I, and synaptophysin. Ctrl, material affinity-purified using His<sub>6</sub>-tagged arfaptin as affinity matrix.

**Figure 3: Interaction of GST-C2B with µ2 is sensitive to high-salt washes but can be restored by calcium and phosphatidylserine**

**A** Binding of µ2 to high-salt washed nucleic-acid free GST-C2B is restored by preincubation with calcium and phosphatidylserine. Untreated or high-salt-washed GST-C2B was preincubated with or without 2 mg/ml liposomes with or without calcium (see experimental procedures). Purified µ2 was added in the presence of 1% triton X-100 and allowed to bind for 1 h at 4°C. Beads were washed and analyzed by SDS-PAGE and staining with Coomassie Blue.

**B** µ2 binding to liposomes. Purified µ2-adaptin (amino acids 157-435) was incubated with or without (-) liposomes containing 20% of either PC, PS, or PI(4,5)P<sub>2</sub> in the presence of either 1 mM CaCl<sub>2</sub> or 5 mM EGTA. Liposome-associated µ2 was analyzed by SDS-PAGE and staining with Coomassie Blue.

**C** Untreated or washed GST-C2B (20 µg protein) preincubated with the indicated lipids in the presence of calcium was used to affinity-purify AP-2 from clathrin coat proteins (100 µg). Samples were analyzed by SDS-PAGE and immunoblotting for clathrin HC and AP-2. 1/10 Std, 10% of the total amount of clathrin and AP-2 added to the assay.

**D** Immobilized untreated or washed GST-C2B (20 µg protein) preincubated with the indicated lipids in the presence of calcium was allowed to bind to recombinant synprint. Samples were washed and analyzed by SDS-PAGE and staining with Coomassie Blue. 1/5 Std, 20% of the total amount of synprint added to the assay.

**Figure 4: Multimerization restores binding of GST-C2B to AP-2 or µ2-adaptin**
(A) Purified high-salt-washed GST-Cys-C2B (5 µg) treated with DTT (red) or copper (II)-1,10-phenanthroline (ox) was analyzed by non-reducing SDS-PAGE and staining with Coomassie Blue.

(B) Reduced or oxidized, high-salt-treated GST or GST-C2B bound to beads was incubated with µ2 (amino acids 157-435) for 1 h at 4°C. Samples were washed and analyzed by SDS-PAGE and staining with Coomassie Blue.

(C) Reduced or oxidized, high-salt-treated GST or GST-C2B bound to beads was incubated with clathrin coat proteins for 1 h at 4°C. Samples were washed and analyzed by SDS-PAGE and immunoblotting for clathrin heavy chain (HC) and AP-2α. Std, 25% of the total amount of coat proteins added to the assay.

(D) Affinity purification of AP-2 using oxidized or reduced GST-Cys-C2B fusion proteins as in (C) and in the presence of the indicated concentrations of the basic synaptotagmin (KR) or a dileucine motif (LL) peptide.

Figure 5: Binding of AP-2 or µ2-adaptin to a GST-fused synaptotagmin peptide.

(A) Affinity purification of AP-2 from rat brain extracts using a GST-fused KR peptide. GST or GST-KR fusion protein (30 µg) immobilized on beads were incubated with triton X-100-extracted rat brain homogenate in the presence of the indicated peptides for 1 h at 4°C. Samples were washed and eluted proteins were analyzed by SDS-PAGE and immunoblotting for AP-2α.

(B) Binding of µ2-adaptin to the GST-fused KR peptide. GST or GST-KR fusion protein (30 µg) immobilized on beads was incubated with µ2 (amino acids 157-435) in the presence of the indicated synthetic peptides (100 µM) for 1 h at 4°C. Samples were washed and eluted proteins were analyzed by SDS-PAGE and staining with Coomassie Blue. 25% Std, 25% of the total amount of µ2 added to the assay.

(C) Mono- or dimeric, high-salt-treated GST or GST-KR bound to beads was incubated with AP-2 'cores' for 1 h at 4°C. Samples were washed and analyzed by SDS-PAGE and staining with Ponceau S (top) followed by immunoblotting against α– and µ2-adaptins (bottom). 25% Std, 25% of the total amount of AP-2 'cores' added to the assay.
Figure 6: Association of recombinant AP-2 cores or Δµ-cores with synaptotagmin peptide.

(A) Multimeric, high-salt-washed GST-Cys-C2B, or GST were incubated with wild-type AP-2 'cores' or 'cores' lacking the c-terminal part of µ2 encompassing the putative synaptotagmin I binding site (Δµ-core). Samples were analyzed by immunoblotting against α-adaptin.

(B) Surface plasmon resonance analysis of the binding of mono- or dimeric synthetic KR peptide to AP-2 cores. K_d values derived from these data are given in brackets.

Figure 7: Grafting the synaptotagmin KR peptide onto tetrameric tailless CD4 facilitates internalization of the chimeric protein from the cell surface.

Internalization of wild-type CD4 or artificially tetramerized CD4tailless-KR or CD4tailless-AA chimeras from the surface of transfected HEK293 cells. Endocytosis was monitored by an antibody feeding assay using anti-CD4 (Q4120) antibodies.

(A) Internalization of CD4 or tetrameric CD4 chimeras in transfected HEK293 cells. Surface staining, Alexa^488 (green); internal, Alexa^594 (red). Scale bar, 10 µm. Note the high levels of surface expression of tetrameric CD4tailless-AA compared to CD4tailless-KR.

(B) Quantification of the data shown in (A). See experimental procedures for details.
Legends to supplementary figures

Supplementary figure 1:

Binding of µ2 (amino acids 157–435) or high-salt washed GST-C2B is restored by preincubation with calcium and phosphatidylserine but not other lipids including phosphoinositides. Untreated or washed GST-C2B was preincubated with 2 mg/ml liposomes and calcium. Purified µ2 was added in the presence of 1% triton X-100 and allowed to bind for 1 h at 4°C. Beads were washed and analyzed by SDS-PAGE and staining with Coomassie Blue. PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; IP3, inositol (1, 4,5)-trisphosphate; TBL, total brain lipids (Folch fraction). 25% Std, 25% of the total amount of µ2 added to the assay.

Supplementary figure 2:

AP-2 was affinity-purified from clathrin coat proteins (100 µg) using untreated, washed, or washed PS (Ca²⁺)-treated GST-C2B. Where indicated, samples contained 200 µM of a peptide (see experimental procedures) corresponding to a functional (Y) or a non-functional (A) tyrosine-based endocytosis motif. Samples were analyzed by SDS-PAGE and immunoblotting for clathrin HC and AP-2. 1/10 Std, 10% of the total amount of clathrin and AP-2 added to the assay.
Figure 1

A

wild-type

mutant

B

Internalization Index (internalized/surface fluorescence)

wild-type

KKAA mutant
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Isabelle Grass, Stefanie Thiel, Stefan Höning and Volker Haucke

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