Internalization signals in synaptotagmin VII utilizing two independent pathways are masked by intramolecular inhibitions

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Summary

The synaptotagmin family of membrane proteins has been implicated in both exocytosis and endocytosis. Synaptotagmin I, a protein containing two tandem C2 domains (the C2A and the C2B) in its cytoplasmic tail, is involved in regulated exocytosis of synaptic vesicles as well as compensatory endocytosis. A related family member, synaptotagmin VII, is involved in multiple forms of regulated exocytosis of lysosomes and secretory granules. In this study we show that the cytoplasmic C2 domains in synaptotagmin VII contain unique internalization signals and regulators of these signals. The C-terminal portion of the C2B is internalized in much the same way as the corresponding region of synaptotagmin I. This signal is tryptophan-based and dynamin and eps15 dependent. In contrast, the C2A contains an unusual internalization signal that is not seen in the C2A of synaptotagmin I. This signal is not based on the homologous tryptophan in its C-terminus. Moreover, internalization of the C2A domain is both dynamin and eps15 independent. Finally, the C2B domain of synaptotagmin VII contains an inhibitory motif that prevents internalization. Endocytic trafficking of synaptotagmin VII is thus governed by these two latent internalization signals, which are concealed by intramolecular inhibition. We propose that endocytosis of synaptotagmin VII is regulated in this way to allow it to couple the processes of regulated exocytosis and compensatory endocytosis.

Key words: Synaptotagmin VII, Clathrin-dependent endocytosis, Clathrin-independent endocytosis, Sorting signals

Introduction

Regulated secretion of proteins and lipids requires a precise balance between fusion of secretory organelles and the recovery of excess membrane from the cell surface. The synaptotagmin family of proteins, previously implicated in calcium-regulated exocytosis, has been suggested to be a key factor in linking these processes.

The 13 current members of the synaptotagmin family of proteins share a unique domain configuration: a short N-terminal region followed by a transmembrane sequence and a long cytoplasmic tail (Perin et al., 1991; Sudhof, 2002) consisting of two tandem C2 domains (the C2A domain and C2B domain) that were originally identified as calcium-binding domains in protein kinase C (Kikkawa et al., 1989). Synaptotagmin I, a neuronal isoform present in synaptic vesicles and secretory granules (Matthew et al., 1981), is the best-studied member of this family. Its role in exocytosis was first suggested by biochemical studies that showed that the C2A domain is responsible for the calcium-dependent association of synaptotagmin I with acidic phospholipids and with syntaxin (Sudhof and Rizo, 1996), although recent in vivo studies have suggested that these particular interactions are not the sole means by which synaptotagmin promotes membrane fusion (Fernandez-Chacon et al., 2001; Robinson et al., 2002). The calcium-dependent interactions of the C2B with phospholipids, or with itself, however, may play a critical role in neurotransmitter release in vivo (Mackler et al., 2002). Additionally, the C2B also participates in a number of calcium-independent interactions with inositol polyphosphates (Fukuda et al., 1994), with the t-SNARE SNAP-25 (Gerona et al., 2000; Schiavo et al., 1997) and with the 'synprint region' of N- and P/Q-type Ca^{2+} channels (Kim and Catterall, 1997; Sheng et al., 1997). Furthermore, mice homozygous for a mutation in the *synaptotagmin I* gene have a severe defect in the fast synchronous, calcium-dependent exocytosis of neurotransmitter (Fernandez-Chacon et al., 2001; Geppert et al., 1994), which is consistent with a role for synaptotagmin I in mediating calcium dependence of synaptic vesicle exocytosis. Similarly, in *Drosophila* mutants, evoked release and calcium-dependent release were severely depressed (Adolfsen and Littleton, 2001). However, synaptotagmin I does not seem to be required for secretory granule exocytosis in neuroendocrine cells (Shoji-Kasai et al., 1992), which suggests that there are molecular differences between synaptic vesicle and secretory granule fusion with the plasma membrane.

Synaptotagmin VII is a close relative to synaptotagmin I and is nearly as abundant as synaptotagmin I. In contrast, however, expression of synaptotagmin VII is not restricted to neurons; rather it is found ubiquitously in many tissue types (Ullrich and Sudhof, 1995). Moreover, synaptotagmin VII is localized to

secretory lysosomes in epithelial cells and fibroblasts (Caler et al., 2001; Martinez et al., 2000). The secretion of these synaptotagmin-VII-containing lysosomes was found to be Ca^{2+} regulated (Martinez et al., 2000), mirroring the regulated secretion of synaptotagmin-I-containing synaptic vesicles (Andrews, 2000; Gerasimenko et al., 2001). In addition, synaptotagmin VII plays a role in dense core vesicle exocytosis in PC12 cells (Shin et al., 2002; Sugita et al., 2001) and in insulin-containing secretory granule exocytosis in pancreatic β-cells (Gao et al., 2000; Gut et al., 2001). Synaptotagmin VII, therefore, contributes to secretory granule and secretory lysosome exocytosis just as synaptotagmin I contributes to synaptic vesicle exocytosis.

The connection between synaptotagmin and endocytosis has only recently begun to be understood. When synaptotagmin I was disrupted in *C. elegans,* a marked depletion of synaptic vesicles was seen at nerve terminals (Jorgensen et al., 1995), which implies a role for synaptotagmin I in vesicular recycling. Moreover, synaptotagmin I contains a conserved high-affinity binding site for AP-2 (Zhang et al., 1994). Blocking action of this site by overexpression of the synprint region of N- and P/Q-type Ca^{2+} channels had an inhibitory effect on transferrin receptor endocytosis in non-neuronal cells (Haucke et al., 2000). In addition, overexpression of a synaptotagmin VII domain for oligomerization inhibits LDL uptake and clathrin-coated pit formation (von Poser et al., 2000). This group of studies implicates synaptotagmins in several forms of endocytosis. Moreover, the interaction of synaptotagmin I with AP-2 is strengthened in the presence of tyrosine-containing domains needed for cargo internalization (Haucke and De Camilli, 1999); an attractive model is that synaptotagmins help recruit clathrin cages in areas of high cargo concentration. They could themselves be cargo for endocytosis or they could remain at the cell surface, passively facilitating coat recruitment.

Synaptotagmin I is itself internalized. Surprisingly, the internalization signal of synaptotagmin I is not identical to and does not require the AP-2-binding site. The internalization signal was found to be in a region of the C2B domain near the C-terminus (Blagoveshchenskaya et al., 1999; Jarousse and Kelly, 2001a). The region of the C-terminus critical for endocytosis was the WHXL motif (N. Jarousse, J. Wilson, D. Arac, J. Rizo and R.B.K., unpublished). Regulation of this internalization signal was shown to be responsible for tissue-specific endocytosis of synaptotagmin I (Jarousse and Kelly, 2001a). Here we show that synaptotagmin VII is not actively internalized in neuronal, fibroblast and epithelial cell types despite having the AP-2-binding site and the WHXL motif, which are important for internalization of synaptotagmin I. Because the internalization signals of synaptotagmin I are latent in some cell types owing to inhibitory elements within the cytoplasmic domain, we looked for internalization signals and inhibitory interactions within the tail of synaptotagmin VII. Out of context, the C-terminal tail (CT) of synaptotagmin VII's C2B was highly endocytosed in a WHXL-dependent manner, identically to the homologous section of synaptotagmin I. In contrast to synaptotagmin I, synaptotagmin VII has a second internalization signal in its C2A domain that lacks both the AP-2-binding site and the previously identified C-terminal WHXL. The homologous WKXL motif in the C2A appeared to play no role in the domain's internalization properties. Although the CT is internalized in a dynamin- and eps15-dependent manner, the C2A takes an unconventional pathway that is independent of both of these proteins. The WHXL-based internalization motif in synaptotagmin VII is normally latent since the C2B does not internalize. The availability of two C2B domains with the same internalization signal but different internalization properties allowed us to map out the region that confers latency in the case of synaptotagmin VII. It was found to reside in the 37 amino acids corresponding to the first two β-strands of synaptotagmin VII's C2B domain. This subdomain was transplantable and conceals or regulates the endocytic signals in the context of either C2B or the full-length synaptotagmin VII protein. Here we have identified two strong endocytic signals in synaptotagmin VII that are concealed by inhibitory elements in the C2B domain. These data suggest that synaptotagmin VII normally acts as a passive facilitator of endocytosis, remaining on the cell surface until special circumstances, as yet unknown, reveal its latent internalization signals.

Materials and Methods

Cell lines

PC12 cells were grown in DMEH-21 media supplemented with 10% horse serum, 5% FCS and penicillin-streptomycin. CHO cells were grown in F12 media supplemented with 10% FCS and penicillin-streptomycin. NRK cells (American Type Culture Collection) were grown in DMEH-21 media supplemented with 10% FCS and penicillin-streptomycin. Phoenix cells (American Type Culture Collection) were grown in DMEH-21 media supplemented with 10% FCS and penicillin-streptomycin.

Reagents and antibodies

The pEGFP plasmid encoding rat synaptotagmin VIIs was provided by Norma Andrews (Yale University, New Haven, CT). The pBMN-Z-I-Neo plasmid was provided by Don Ganem (University of California at San Francisco, San Francisco, CA).

The monoclonal antibody against the lumenal domain of the human CD4 (clone Q4120) was obtained from the Medical Research Council AIDS Reagents Program (National Institute for Biological Standards and Control) for use in internalization assays. A second monoclonal antibody against the lumenal domain of CD4 (Pharmigen, clone RPA-T4) was used for immunofluorescence and flow cytometry.

Constructs

For the CD4-synaptotagmin constructs, a CD4 fragment (corresponding to residues 1-426) encoding the lumenal, transmembrane and 12 amino acids of the cytoplasmic region of the human CD4 was amplified by PCR from pBMN-Syt 1 (Jarousse and Kelly, 2001a). The primers were chosen so that the CD4 coding region was downstream of a *Bam*HI restriction site and upstream of a *Bst*BI restriction site followed by a stop codon and *Sal*I restriction site. This fragment was digested with *Bam*HI and *Sal*I and inserted into the corresponding sites in pBMN-Z-I-Neo to generate pBMN-CD4-Tailless. The cytoplasmic domains of synaptotagmin I were amplified by PCR from the pBMN-Syt 1 plasmid to generate the following fragments: C2A-C2B (encoding residues 95-421), C2A (residues 95-265), C2B (residues 266-421), and CT (residues 393-421). Similarly, the cytoplasmic domains of synaptotagmin VII were amplified by PCR from the pEGFP-Syt VIIs plasmid to generate the following fragments: C2A-C2B (encoding residues 98-403), C2A (residues 98-260), C2B (residues 261-403) and CT (residues 387-403). The forward primers were flanked with a *Bst*BI restriction site, and the

reverse with a stop codon (for the C2A fragments only) and a *Sal*I restriction site. The PCR products were digested and ligated to the corresponding sites of pBMN-CD4-Tailless to generate in-frame CD4/synaptotagmin fusions. The Syt 7 C2A W253A, Syt 7 CT W398A, and Syt 1 CT W404A mutants were generated using a QuikChange site-directed mutagenesis kit (Stratagene). The C2B chimeras were generated in a two-step PCR-based cloning strategy. First, the C2B fragment was amplified using an outer primer complementary to a single synaptotagmin sequence and a chimeric primer composed of half Syt 1 sequence and half Syt 7 sequence surrounding the junction point. The corresponding C2B fragments were then mixed together and amplified using the opposing synaptotagmin outer primers alone to generate a chimeric C2B construct. The outer forward primers were flanked with a *Bst*BI restriction site, and the outer reverse with a *Sal*I restriction site. The PCR-generated chimeras were digested and ligated to the corresponding sites of pBMN-CD4-Tailless. The chimeric construct compositions are detailed in the Fig. 7 legend. All constructs were verified by sequencing.

Transfections and retroviral infections

For retroviral infections, we used a vector derived from pBMN-Z-I-Neo in which the *LacZ* gene was deleted (fragments *Bam*HI-*Sal*I) and replaced by our genes of interest (containing the CD4/synaptotagmin ORFs). The vector contains the internal ribosome entry site of the encephalomyocarditis virus upstream of the neomycin resistance gene. This permits both the gene of interest and the neomycin resistance gene to be translated from a single bicistronic mRNA. Using this method, nearly all surviving colonies will stably express the gene of interest after selection with G418. Expression of the bicistronic mRNA is controlled by the 5′ viral LTR promoter (full-length Moloney LTR).

The Phoenix cells were transfected with the different CD4-synaptotagmin constructs using Fugene-6 transfection reagent (Roche). On transfection of the vectors, the Phoenix-packaging cell line produces replication-defective viral particles that were used for stable gene transfer and expression in PC12, CHO and NRK cells. Virus-containing supernatants were filtered through a low binding protein 0.45 μm filter (Pall Corporation) 48 hours after transfection, supplemented with 4 μg/ml of hexadimethrine bromide (Sigma-Aldrich) and used to infect PC12, CHO or NRK cells. After 48-72 hours, 400 μg/ml of G418 was added. Seven to 10 days after infection, colonies were pooled and propagated in culture in the presence of 400 μg/ml of G418. Cells were treated for 21 hours before the experiments with 500 nM of trichostatin A to enhance expression of the constructs.

Internalization assays using iodinated antibodies

50 μg of Q4120 antibody was iodinated on iodogen-coated tubes (Pierce Chemical Co.) as described previously (Clift-O'Grady et al., 1998). Cells were plated on collagen and poly-D-lysine-coated 12-well plates two days before the assay. Cells were incubated for 1 hour at 4°C with 100 ng/ml of ^{125}I-Q4120 in DMEH-21 media supplemented with 1% BSA and 10 mM HEPES, pH 7.4. Unbound antibody was removed by extensive washes. Cells were next incubated at 37°C for 10 minutes to allow endocytosis and then returned to 4°C. Antibodies remaining at the cell surface were removed by two 10 minute acid-stripping washes at 4°C in PBS/BSA supplemented with 30 mM glycine and adjusted to pH 2.4. Acid-resistant antibody was collected by lysing the cells in 2 M NaOH. The fraction of antibody internalized was calculated by dividing the acid-resistant radioactive cpm by the sum of acid-resistant and -accessible cpm and averaging over the samples taken in triplicate. A background of acid-resistant counts in cells kept at 4°C was subtracted from each value, and the error bars depict s.e.m.

Immunofluorescent microscopy

PC12 cells were plated onto eight-well collagen and poly-D-lysine-coated slides at various densities two days before the slides were processed. For uptake experiments, the cells were chilled on ice and were incubated for 1 hour at 4°C with 1 μg/ml anti-CD4 monoclonal antibody (Pharmigen, clone RPA-T4) in DMEH-21 media supplemented with 1% BSA and 10 mM HEPES, pH 7.4. Unbound antibody was removed by extensive washes. Cells were next incubated at 37°C for 10 minutes to allow endocytosis and then returned to 4°C. After washing in ice-cold PBS, cells were fixed in 4% paraformaldehyde and quenched in PBS, 25 mM glycine. Next, the cells were permeabilized and blocked for 1 hour in 2% BSA, 1% fish skin gelatin and 0.02% saponin in PBS (blocking solution). Finally, the cells were stained for 1 hour at room temperature with secondary antibody, 10 μg/ml AlexaFluor 488 goat-anti-mouse IgG (Molecular Probes) in blocking solution. After several washes, slides were mounted in Movial and viewed with a 100× oil immersion lens on a Zeiss Axioscope.

Flow cytometry analysis

PC12 cells stably expressing synaptotagmin VII C2A-C2B, C2A or CT were transiently transfected with expression vectors using Lipofectamine 2000 (Life Technologies). A single well of a six-well plate was transfected per sample two days before flow cytometry analysis. The pIRES2-EGFP expression vectors encoded either endocytic regulators [the wild-type or dominant negative versions of dynamin (Schmidlin et al., 2001) or eps15 (Benmerah et al., 1999)] or the regulatory fragment of interest of the C2B. In the case of the eps15 constructs, the coding region was fused directly to EGFP. However, in the case of the dynamin constructs and C2B fragments, the vector also contains an internal ribosome entry site of the encephalomyocarditis virus between the multiple cloning site (MCS) and the enhanced green fluorescent protein (EGFP) coding region. Both vector architectures permit the gene of interest (cloned into the MCS) and the *EGFP* gene to be translated and allows for the identification, by flow cytometry, of transiently transfected mammalian cells expressing EGFP and the protein of interest. The cells were harvested, labeled at 4ºC for 1 hour with 1 μg/ml anti-CD4 monoclonal antibody (Pharmigen, clone RPA-T4) in PBS, 1% BSA. After washing, surface-bound antibody was visualized by addition at 4°C for 30 minutes of 1 μg/ml phycoerythrin-conjugated goat $F(ab')_2$ anti-mouse IgG antibody (Caltag) in PBS, 1% BSA. After washing and resuspending in PBS, cells were analyzed with a Becton Dickinson FACScalibur. The data were collected in a logarithmic mode, and the mean of fluorescence intensity was calculated.

Structural analysis

The structural model of the synaptotagmin VII C2B was constructed using the Swiss-Model program (www.expasy.ch/swissmod/SWISS-MODEL.html) basing the homology-modeling on the published structure of synaptotagmin I C2B (Fernandez et al., 2001). The figure was then prepared using the WebLab Viewer Lite 3.2 software.

Results

Synaptotagmin VII is not endocytosed in PC12 cells or CHO cells

Previous studies have shown that the synaptic vesicle protein synaptotagmin I can be endocytosed in PC12 cells but not CHO cells (Jarousse and Kelly, 2001a), presumably because CHO cells lack the machinery required for synaptic vesicle recycling. Since both synaptotagmin I and synaptotagmin VII are involved in regulated exocytosis events, we wanted to

determine if synaptotagmin VII was also subject to cell-type-specific endocytosis. In order to test this possibility, we generated fusion constructs between the CD4 lumenal and transmembrane domains and the synaptotagmin full-length cytoplasmic domain (C2A-C2B). These proteins were stably expressed in PC12, CHO and NRK cells by retroviral infection. They were subsequently tested for internalization by labeling cell surface synaptotagmin VII with ^{125}I-Q4120, anti-CD4, at 4°C and allowing internalization at 37°C for 10 minutes. When tested in PC12 cells, we did not detect significant internalization of synaptotagmin VII relative to synaptotagmin I (Fig. 1A). Since synaptotagmin VII is on the cell surface of PC12 cells (Sugita et al., 2001) but is intracellular in epithelial normal rat kidney (NRK) cells (Martinez et al., 2000) and in fibroblastic Chinese hamster ovary (CHO) cells (Caler et al., 2001), we examined endocytosis in these two cell types showing an internal organellar staining and known to demonstrate regulated exocytosis. Nevertheless, neither synaptotagmin I nor synaptotagmin VII was endocytosed in the case of CHO cells (Fig. 1B) or NRK cells (data not shown). To determine if endocytosis could be activated by stimulation of the exocytic pathway, we increased intracellular calcium levels in the cells. Towards this end, we used 10 μM ionomycin (Martinez et al., 2000) either before or during the 37°C incubation in CHO cells or 90 mM K^+ depolarization in PC12 cells. None of the stimuli tested had detectable effects on

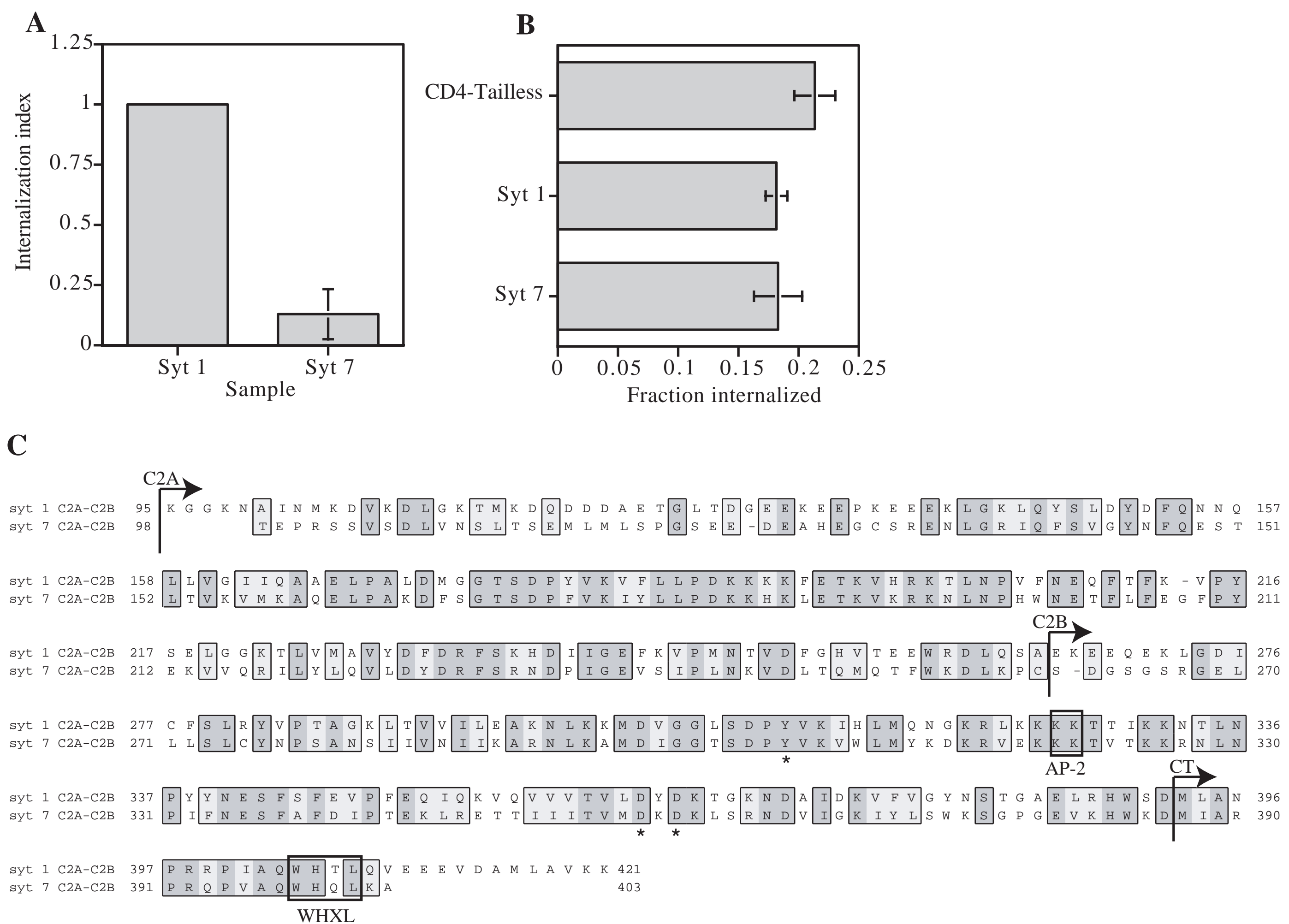


Fig. 1. Synaptotagmin VII is not endocytosed in PC12 cells or CHO cells. The CD4-C2A-C2B constructs of synaptotagmin I (Syt 1) and synaptotagmin VII (Syt 7) were tested for their ability to be endocytosed. The cytoplasmic domains corresponded to residues 95-421 of full-length synaptotagmin I and residues 98-403 of full-length synaptotagmin VII. (A) The internalization was tested by surface labeling the cells for 1 hour at 4°C with ^{125}I-Q4120, directed against the CD4 epitope, and next incubating at 37°C for 10 minutes to allow endocytosis before returning the cells to 4°C. Internalized ^{125}I-Q4120 was determined by acid stripping the remaining surface label and lysing cells to quantify internalized counts. The fraction internalized was calculated by dividing the internal counts by the total cell associated counts (surface counts plus internalized counts). A background of cells kept at 4°C was subtracted from this value. This fraction internalized was then converted to an internalization index by subtracting the non-specific internalization of the CD4-Tailless construct and by normalizing to the extent of internalization of the Syt 1 construct in PC12 cells. (B) CHO cells stably expressing the CD4-C2A-C2B constructs were also analyzed using this internalization assay; the internalization is compared in terms of fraction internalized relative to the CD4-Tailless construct. (C) The C2A-C2B domains of synaptotagmins I and VII are aligned to show conserved sequence elements including the AP-2-binding site and the C-terminal WHXL. The asterisks (*) indicate Ca^{2+}-binding residues known to influence dimerization and internalization of synaptotagmin I, and the residue numbers correspond to the position of the amino acids within the full-length protein.

synaptotagmin VII's ability to endocytose (data not shown). Thus although the synaptotagmins have been implicated in endocytosis in fibroblast-like cells (Haucke et al., 2000; von Poser et al., 2000), synaptotagmin VII does not itself have internalization signals that are active under the conditions tested.

The C-terminal and C2A domains of synaptotagmin VII contain functional internalization signals

Failure to be internalized is not an artifact of transfection, but is due to the inaccessibility of an internalization domain. Synaptotagmin VII has cytoplasmic domains very similar to those of synaptotagmin I, in particular having the AP-2-binding site, the WHXL motif and the calcium-binding/oligomerization residues (Fig. 1C). To identify any latent internalization signals, we generated fusion proteins that isolated the individual cytoplasmic domains of synaptotagmin VII. Such a signal was predicted to be in the C-terminal fragment of synaptotagmin VII (CT 7), a segment of the C2B domain that includes the WHXL motif found to be a strong internalization signal in synaptotagmin I (N. Jarousse, J. Wilson, D. Arac, J. Rizo and R.B.K., unpublished). This region of synaptotagmin VII had as strong an internalization signal as the corresponding domain in synaptotagmin I in PC12 cells (Fig. 2A) and CHO cells (data not shown). Likewise, the

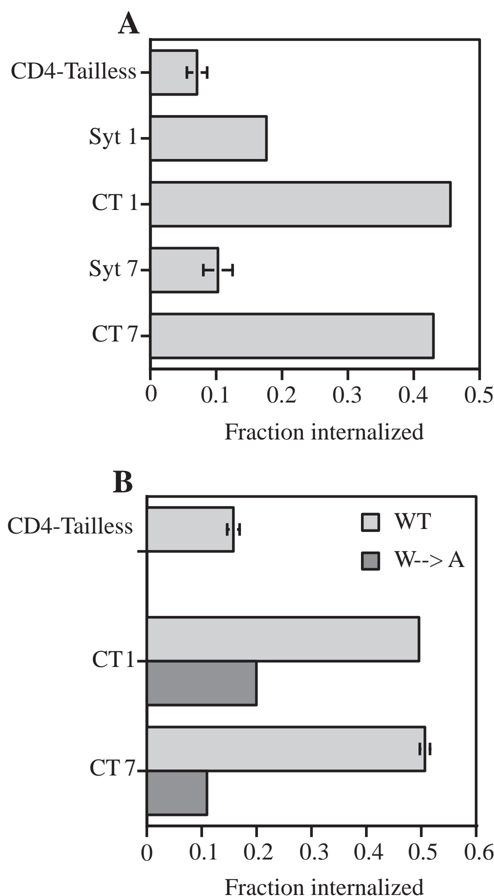


Fig. 2. The CT of synaptotagmin VII can be internalized in a tryptophan-dependent manner. (A) The CD4-CT constructs of synaptotagmin I (residues 393-421, CT 1) and synaptotagmin VII (387-403, CT 7) were tested for their ability to be endocytosed in PC12 cells using the previously described internalization assay. (B) When the tryptophan residue with the C-terminal WHXL is mutated to an alanine in either CT1 (W404A) or CT 7 (W398A), the internalization in PC12 cells is abolished.

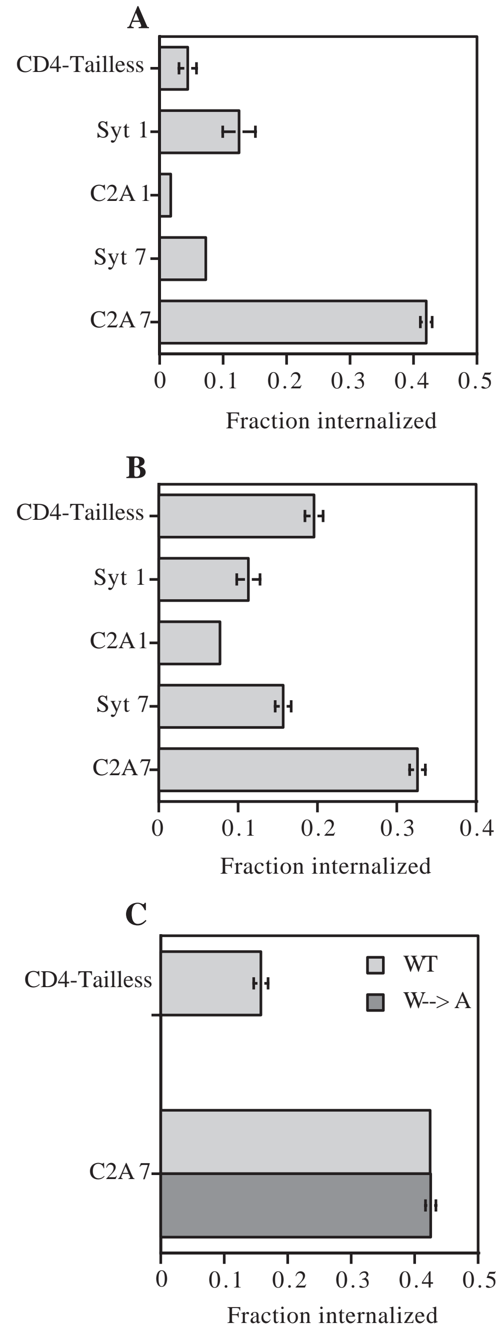


Fig. 3. The C2A of synaptotagmin VII is internalized in a cell-type-independent and tryptophan-independent manner. Using the previously described internalization assay, the CD4-C2A constructs of synaptotagmin I (residues 95-265, C2A 1) and synaptotagmin VII (residues 98-260, C2A 7) were tested. PC12 cells (A) and CHO cells (B) stably expressing the constructs were both analyzed by this method. (C) A point mutant changing the tryptophan residue of C2A 7's WKXL (W253A) to an alanine did not affect the strong endocytosis of C2A 7 in PC12 cells.

internalization of the synaptotagmin VII CT was dependent on the presence of the analogous tryptophan within the WHXL, as an alanine mutant failed to internalize at a rate above background in PC12 cells (Fig. 2B). Thus, synaptotagmin VII has the same internalization motif as is used by synaptotagmin I, but it is completely latent in PC12 cells.

Next, the C2A domain of synaptotagmin VII (C2A 7) was tested for its ability to be internalized in PC12 cells. Surprisingly, although the corresponding domain had no endocytic properties in synaptotagmin I (Blagoveshchenskaya et al., 1999; Jarousse and Kelly, 2001a), this domain in synaptotagmin VII contained a potent internalization signal (Fig. 3A). Moreover, the signal within this domain appears to be a universal signal, as demonstrated by the fact that C2A is internalized in CHO cells as well (Fig. 3B). Notably, this domain, despite being homologous to the C2B domain, does not contain the polybasic region known to bind AP-2. It does, however, contain a sequence at its C-terminal end that is similar to the WHXL in location and sequence. We next mutated the tryptophan in the C2A's WKXL sequence to an alanine and tested this construct for its ability to be endocytosed. In this case, the tryptophan is not involved in the endocytosis of this domain (Fig. 3C).

We verified the internalization of the C2A domain by a morphological assay of endocytosis. When PC12 cells were incubated with anti-CD4 antibody at 4°C, the labeled fusion proteins remained at the cell surface (Fig. 4A,C,E). When the temperature was increased to 37°C for 10 minutes, the CD4-C2A-C2B synaptotagmin VII construct continued to remain at the plasma membrane (Fig. 4B). However, when the synaptotagmin VII CT- and C2A-expressing cells were moved to 37°C, these proteins were taken up into internal compartments (Fig. 4D,F). This confirmed that both the CT and C2A domains of synaptotagmin VII contain strong internalization signals.

The C-terminus and C2A domains are internalized by different mechanisms

Endocytosis mechanisms can be subdivided according to their sensitivity to dominant-negative inhibitors. Although the CT WHXL signal in synaptotagmin I is not a typical internalization signal, it is internalized by a conventional AP-2- and dynamin-dependent pathway (N. Jarousse, J. Wilson, D. Arac, J. Rizo and R.B.K., unpublished). By analogy, the CT of synaptotagmin VII should behave in a similar way. To

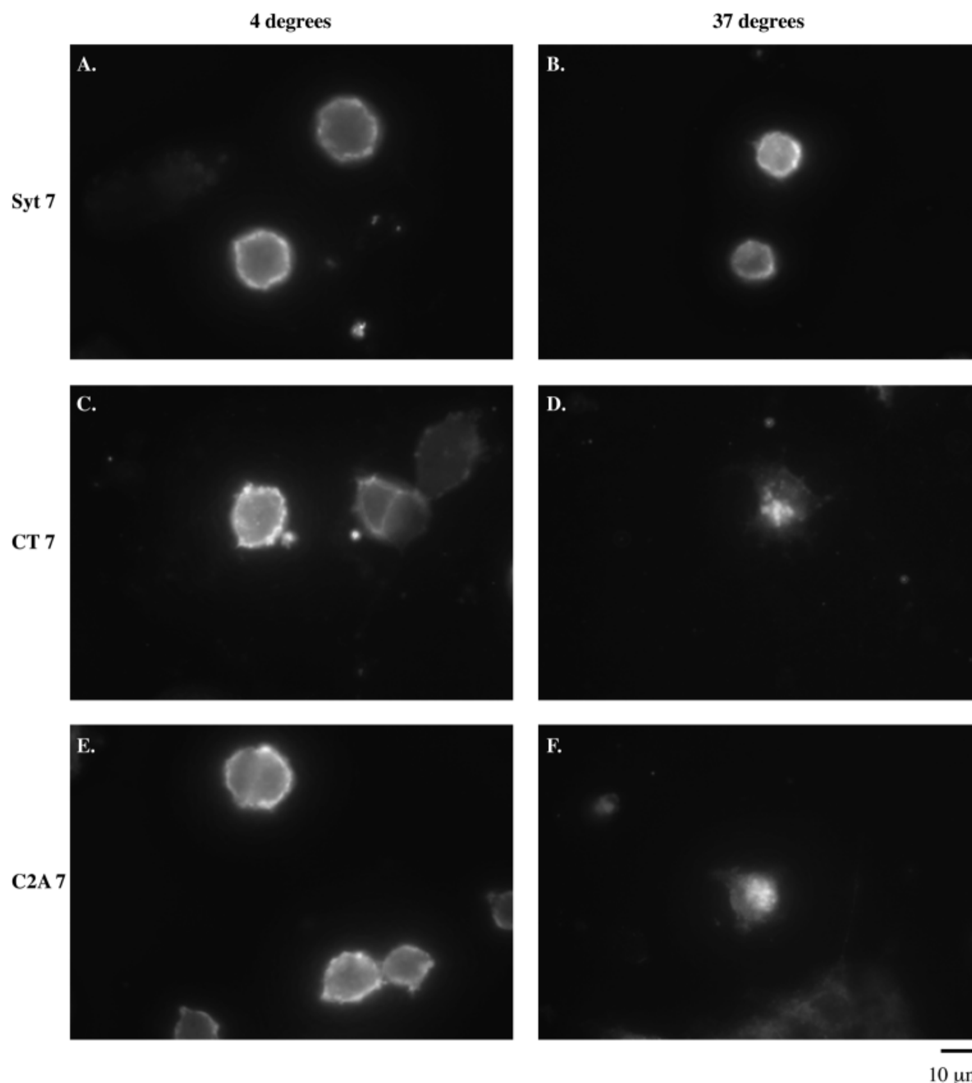
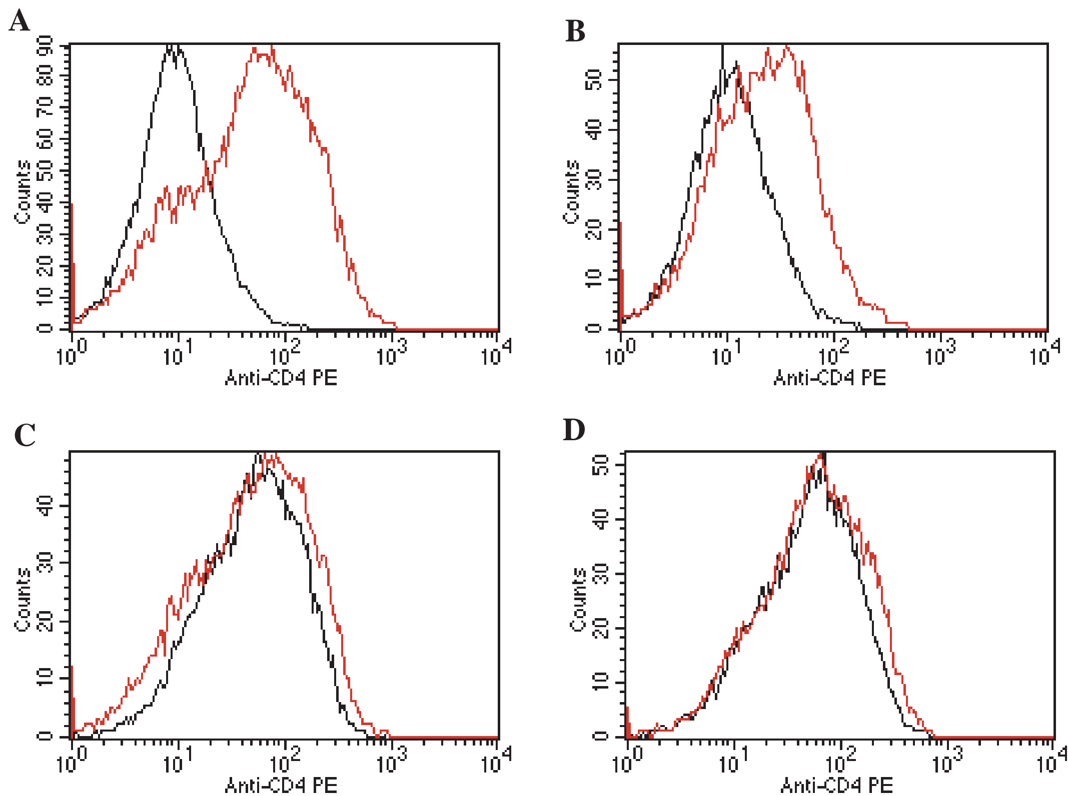


Fig. 4. The CT and C2A of synaptotagmin VII are taken up into an internal compartment in PC12 cells. A morphological uptake assay was used to verify the results seen with the radioactive internalization assays. In this case, PC12 cells stably expressing Syt 7 (A,B), CT 7 (C,D) and C2A 7 (E,F) were plated on eight-well slides, surface stained with anti-CD4 antibody at 4°C (A,C,E) and moved to 37°C for 10 minutes (B,D,F). Cells were fixed, and immunofluorescence microscopy was carried out.

substantiate this hypothesis, we used specific inhibitors of traditional clathrin-mediated endocytosis to analyze their effect on the trafficking of both the CT and C2A domains of synaptotagmin VII. We used both functional and dominant-negative versions of dynamin or eps15, tagged by coexpression with or fusion to GFP, transiently transfected into stable cells expressing either the synaptotagmin VII CT fragment or the C2A domain. The dynamin K44E mutant, mutated in its GTP-binding pocket, acted in a dominant-negative fashion to inhibit dynamin-dependent endocytosis (Herskovits et al., 1993). Likewise, the eps15 fragments used were found to retain their endocytic ability when the AP-2-binding domain was deleted (fragment D3Δ2) but acted as dominant-negative inhibitors of AP-2-dependent endocytosis when the EH domain was deleted (EΔ95/295) (Benmerah et al., 1999). We measured both the cell surface CD4 staining for the synaptotagmin VII domains and the GFP fluorescence for expression of the endocytic regulator by fluorescence-activated cell sorting. In the event that the construct's internalization was inhibited, we expected to see an increased amount of cell surface staining in high-GFP-expressing cells. In fact we found that, in agreement with the result for the synaptotagmin I CT, the CT of synaptotagmin VII had increased levels at the plasma membrane when transfected with dominant-negative dynamin in comparison with wild-type dynamin (Fig. 5A). Furthermore, use of the eps15 EΔ95/295 mutant also resulted in increased cell surface expression of synaptotagmin VII CT compared to use of eps15 D3Δ2 (Fig. 5B). These conditions potently inhibited internalization of the CT as the dominant negatives resulted in a 6.8- and 2.0-fold increase in cell surface staining respectively (Fig. 5E).

When the parallel experiments were conducted using the synaptotagmin VII C2A, the outcome was dramatically



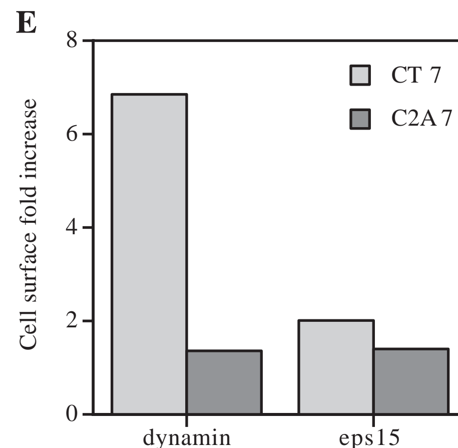


Fig. 5. The CT and C2A of synaptotagmin VII are internalized by different pathways in PC12 cells. PC12 cells stably expressing either CT 7 (A,B) or C2A 7 (C,D) were transiently transfected with wild-type dynamin or dominant-negative dynamin K44E (A,C) or with control eps15 epsD3Δ2 or dominant-negative eps15 epsΔ95/295 (B,D). The x-axis of the histograms shows the level of cell surface staining in each case. The curve for the wildtypes or controls are shown in black, and the curves for the dominant-negative mutants are shown in red. The mean of this value was taken and used to generate the calculated factor 'cell surface fold increase' by dividing the mean surface staining in the dominant negative by the mean of the wildtype or control case (E).

different. Specifically, neither the K44E dynamin nor the eps15 EΔ95/295 led to increased amounts of cell surface synaptotagmin VII C2A (Fig. 5C-E). This places the internalization of synaptotagmin VII's C2A domain into the unusual category of AP-2- and dynamin-independent endocytosis.

A C2B subdomain is inhibitory to synaptotagmin VII endocytosis

Although the entire cytoplasmic domain cannot be internalized, the CT region of synaptotagmin VII has the ability to be endocytosed when removed from its normal environment as part of the C2B domain. When the structure of synaptotagmin I's C2B domain was solved (Fernandez et al., 2001), it became apparent that the CT was an integral part of the eight-stranded β-sandwich structure of the C2B domain itself. We, therefore, asked if the presence of the C2A domain affected recognition of the C2B's internalization signal. In contrast to synaptotagmin I, the isolated C2B of synaptotagmin VII did not retain an enhanced ability to be endocytosed (Fig. 6). This suggests the existence of a region within the C2B preventing internalization of synaptotagmin VII by its tryptophan-based motif.

To more narrowly map the region within the C2B that was inhibitory to the tryptophan-based motif, we designed chimeric constructs between the C2B domains of synaptotagmin I and VII. Because these chimeras were being generated within a single domain, we took care to choose chimeric junction points that would least disrupt the domain's structure. Using the structure of synaptotagmin I's C2B (Fernandez et al., 2001) and the homology between the C2Bs of synaptotagmin I and VII, we generated chimeric C2Bs where the transition points were within the flexible loops, which were predicted to tolerate more motion than other regions of the domain. Moreover, these switches were in areas of highly conserved amino-acid sequence, so the resulting deformation should have been minimal. Chimeras were made with β-strands of the eight-stranded β-barrel replaced in pairs.

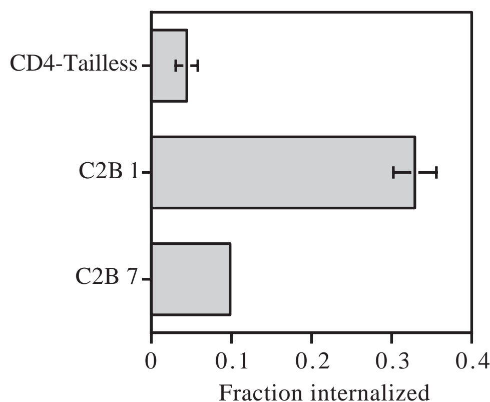


Fig. 6. The C2B of synaptotagmin VII is not internalized in PC12 cells. PC12 cells were generated that stably expressed CD4-C2B constructs for synaptotagmin I (residues 266-421, C2B 1) and synaptotagmin VII (residues 261-403, C2B 7). These cells were examined by the previously described radioactive internalization assay.

The resulting chimeras fell into two categories: a synaptotagmin VII C2B with increasing numbers of N-terminal synaptotagmin-I-derived β-strands, and the inverse series. These proteins were subsequently tested for their ability to be endocytosed. The C2B series with increasing contributions from synaptotagmin I clearly showed that when the first two β-strands of synaptotagmin VII were replaced with those of synaptotagmin I, the inhibition was also removed (Fig. 7A). Inversely, when the C2B of synaptotagmin I gained the first two β-strands of synaptotagmin VII, it also gained the inhibitory property of synaptotagmin VII (Fig. 7B). Therefore, this inhibitory subdomain of synaptotagmin VII is transplantable and is in the first two strands of the β-sandwich. To test the possibility that the synaptotagmin VII subdomain was binding to an inhibitory factor acting through the C2B, we asked if the subdomain could be overexpressed to titrate out the putative inhibitory factor. The prediction was that overexpression could overwhelm the inhibitory machinery to activate endocytosis of synaptotagmin VII. As a control, we also overexpressed GFP alone or the cognate region of synaptotagmin I and then measured cell surface levels of synaptotagmin VII by fluorescence-activated cell sorting. The overexpression of the inhibitory fragment of synaptotagmin VII did not relieve the inhibition of endocytosis on CD4-C2A-C2B synaptotagmin VII, as its levels at the cell surface remained high (data not shown).

Discussion

Our finding that the full-length cytoplasmic domain of synaptotagmin VII is not internalized suggests that any role it plays in the general process of endocytosis (Haucke et al., 2000; von Poser et al., 2000) is passive, facilitating internalization without being internalized itself. Synaptotagmin VII may recruit AP-2 (Li et al., 1995) and cargo proteins to sites of endocytosis without itself being concentrated into coated pits.

A paradox, however, is that synaptotagmin VII has internalization signals, but they are latent. Synaptotagmin I has one of the internalization signals, and it too is latent in cells that do not recycle synaptic vesicles. Synaptic vesicle recycling is a form of compensatory endocytosis (Jarousse and Kelly, 2001b) by which the membranes of secretory vesicles are rapidly retrieved in a homeostatic mechanism that preserves cell surface area. Because synaptotagmin VII is involved in a number of regulated exocytosis events (Caler et al., 2001; Gao et al., 2000; Gut et al., 2001; Martinez et al., 2000; Reddy et al., 2001; Shin et al., 2002), one might expect to find that its internalization signal is latent until exocytosis is activated. We did not succeed in finding conditions or cell types in which synaptotagmin VII is efficiently internalized. It is possible that synaptotagmin VII's endocytic signals are only activated in situations where the lysosomal pool is massively depleted. Similarly, a wound healing mechanism might not automatically induce compensatory endocytosis until an activating signal communicates completion of the healing process.

An additional interesting aspect of the internalization signals is the unconventional nature of the C2A signal. We found that the internalization of this domain did not require either dynamin or eps15, whereas the internalization of synaptotagmin VII's CT fell into the more typical category of

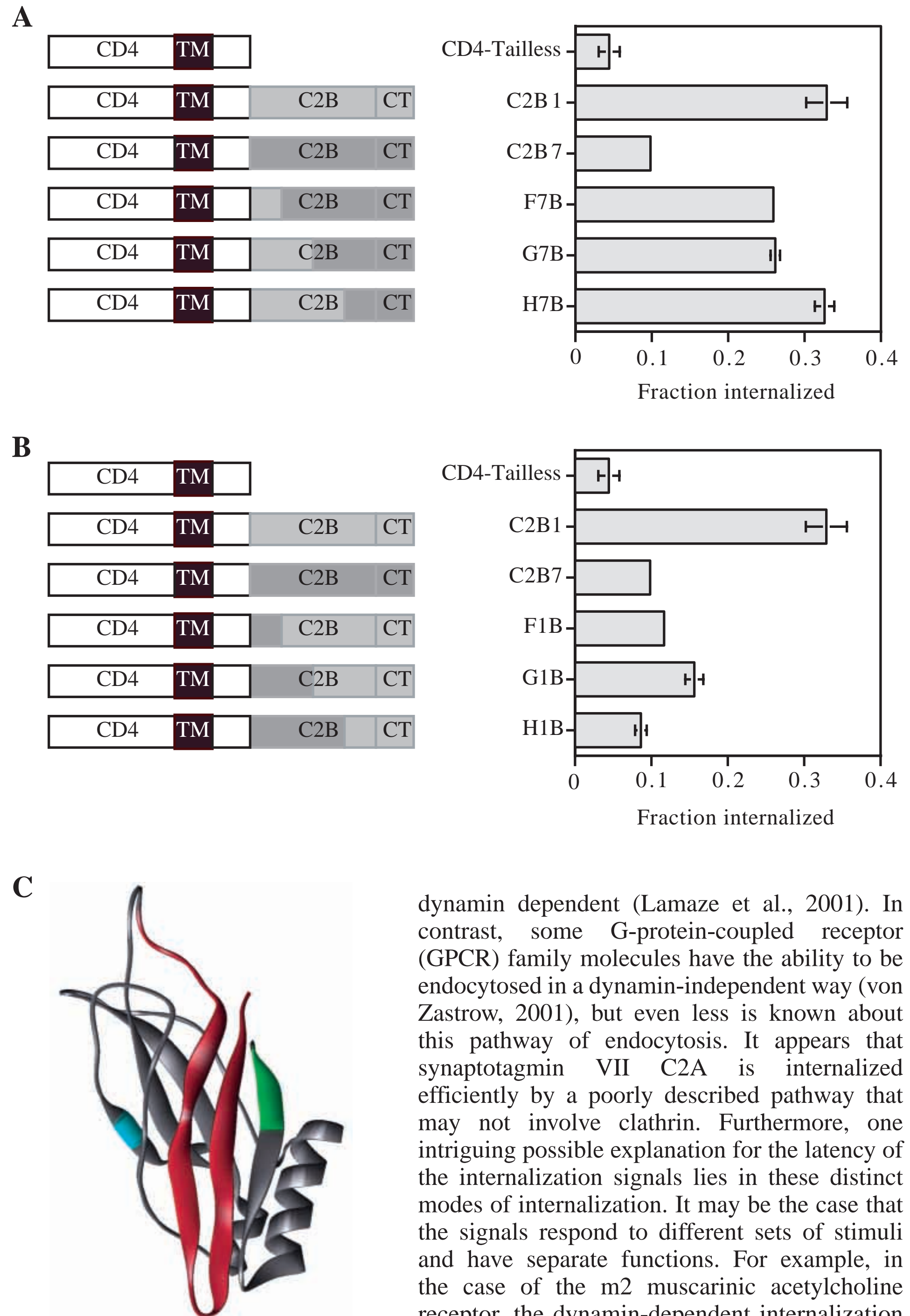


Fig. 7. The C2B of synaptotagmin VII contains a dominant and transplantable inhibitory subdomain. Chimeric constructs were generated that replaced β-strands of the eight-stranded β-barrel in pairs. The resulting chimeras fell into two categories: a synaptotagmin VII C2B with (A) increasing numbers of N-terminal synaptotagmin-I-derived β-strands [7B series (F7B-H7B)] and (B) the inverse series [1B series (F1B-H1B)]. The construct compositions are as follows: F7B (synaptotagmin I residues 266-303 fused to synaptotagmin VII residues 298-403), G7B (synaptotagmin I residues 266-334 fused to synaptotagmin VII residues 329-403), H7B (synaptotagmin I residues 266-369 fused to synaptotagmin VII residues 364-403), F1B (synaptotagmin VII residues 261-297 fused to synaptotagmin I residues 304-421), G1B (synaptotagmin VII residues 261-328 fused to synaptotagmin I residues 335-421), H1B (synaptotagmin VII residues 261-363 fused to synaptotagmin I residues 370-421). PC12 cells expressing these chimeras were subsequently studied for their capacity to endocytose the proteins with the endocytosis assay. (C) A model of the synaptotagmin VII C2B domain depicts the relative positions of the inhibitory region (red), the AP-2-binding site (cyan) and the WHXL motif (green).

dynamin- and eps15-dependent endocytosis. When IL-2 receptors are internalized by an eps15-independent pathway (Lamaze et al., 2001) they are concentrated into detergent-resistant membrane subdomains that do not colocalize with clathrin-coated pits. Thus, the C2A domain could allow synaptotagmin VII to be internalized by an AP-2-independent pathway, which might even allow targeting to a different intracellular compartment. However, the route of internalization for IL-2 receptors is likely to be distinct from that of the C2A of synaptotagmin VII since it appears to be dynamin dependent (Lamaze et al., 2001). In contrast, some G-protein-coupled receptor (GPCR) family molecules have the ability to be endocytosed in a dynamin-independent way (von Zastrow, 2001), but even less is known about this pathway of endocytosis. It appears that synaptotagmin VII C2A is internalized efficiently by a poorly described pathway that may not involve clathrin. Furthermore, one intriguing possible explanation for the latency of the internalization signals lies in these distinct modes of internalization. It may be the case that the signals respond to different sets of stimuli and have separate functions. For example, in the case of the m2 muscarinic acetylcholine receptor, the dynamin-dependent internalization is agonist-induced, whereas the dynamin-independent internalization is continuous (Pals-Rylaarsdam et al., 1997). Similarly, the two internalization signals in synaptotagmin VII could be utilized in different contexts.

The latency of the tryptophan-based internalization signal within synaptotagmin VII is unusual because it remains concealed owing to active inhibition by a dominant subdomain of the C2B. Other cell surface proteins also have latent endocytic signals. In the case of GPCRs, the receptors remain at the cell surface until they are activated by ligand binding. This binding triggers receptor phosphorylation and subsequent

recruitment of β-arrestin to bridge the molecule to the endocytic machinery (Ferguson, 2001). Likewise, for receptor tyrosine kinases, ligand binding causes the receptor to become covalently modified by autophosphorylation and, in some cases, ubiquitination before being recognized by the endocytic machinery (Clague and Urbe, 2001). The common feature of these endocytic events is that the signaling molecules must first undergo a covalent modification that allows recruitment of machinery to couple the signaling event to the trafficking event. At the synapse, endocytic proteins are dephosphorylated after calcium-dependent transmitter release to allow them to participate in endocytosis associated with synaptic vesicle recycling (Lauritsen et al., 2000; Slepnev et al., 1998). In addition, synaptotagmin I itself undergoes calcium-dependent phosphorylation by CaMKII, which strengthens its association with the exocytic SNARE machinery of syntaxin and SNAP 25; subsequent dephosphorylation weakens this interaction and could potentially promote endocytosis of synaptotagmin I (Verona et al., 2000). This cyclic regulation of exocytic and endocytic versions of synaptotagmin is supported by the fact that the phosphorylated form of synaptotagmin I is found in exocytosis-competent synaptic vesicles but not in clathrin-coated vesicles that presumably have arisen by endocytosis (Hilfiker et al., 1999). It is possible that the C2B of synaptotagmin VII could exert its inhibitory effects by either activating the phosphorylation of synaptotagmin VII or by preventing its later dephosphorylation. Because of the relative proximity of the inhibitory region to the WHXL motif (Fig. 7C), a phosphorylation event in the inhibitory region could cause a conformational change that would render the WHXL inaccessible. Alternatively, recruitment of a protein factor to this region could sterically prevent recognition of the WHXL by the endocytic machinery. Inhibition by phosphorylation would, however, require phosphorylation sites within the C2B domain itself, whereas all previously identified sites are in the C2A domain.

Synaptotagmin VII endocytosis is governed by a complex collection of activating and inhibitory signals that, under most conditions, retain synaptotagmin VII at the plasma membrane. It is our model that synaptotagmin VII acts at this site as a passive facilitator of endocytosis where it activates endocytosis of cargo molecules other than itself. Under certain, as yet unidentified, conditions, it can be internalized by two different endocytic pathways. Neuronal synaptotagmins have adapted regulated endocytosis for use in the efficient recycling of synaptic vesicle membranes. We hypothesize that a similar activating event takes place to make synaptotagmin VII's signals available for endocytosis in a context-dependent fashion.

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