

# The AP2 binding site of synaptotagmin 1 is not an internalization signal but a regulator of endocytosis

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One characteristic linking members of the synaptotagmin family to endocytosis is their ability to bind the heterotetrameric AP2 complex via their C2B domain. By using CD4/synaptotagmin 1 chimeras, we found that the internalization signal of synaptotagmin 1 lies at the extreme COOH-terminus of the protein and can function in the absence of the C2B domain that contains the AP2 binding site. However, although not essential for internalization, the C2B domain of synaptotagmin 1 appeared to control the recognition of the internalization motif. By mutagenesis, two sites have been identified that modify regulation by the C2B domain in the neuroendocrine PC12 cell line. Mutation of a dilysine motif in the  $\beta$

sandwich core of the domain eliminates endocytosis. This site is known to be a site of protein–protein interaction. Mutations in the calcium binding region, or in its close proximity, also affect internalization in PC12 cells. In fibroblasts, the C2B domain inhibits the COOH-terminal internalization signal, resulting in an absence of internalization in those cells. Thus, internalization of synaptotagmin 1 is controlled by the presence of a latent internalization signal in the COOH-terminal region and a regulatory region in the C2B domain. We propose that internalization of synaptotagmin 1 is regulated in this way to allow it to couple the processes of endocytosis and calcium-mediated exocytosis in cells of the neuroendocrine lineage.

## Introduction

Several cell surface proteins have conserved internalization signals in their cytoplasmic domains. Such proteins become cargo for the endocytotic machinery of cells when their internalization signals are recognized by the  $\mu$  or the  $\beta$  subunit of heterotetrameric AP2 adaptors, which in turn bind clathrin and several accessory proteins (Kirchhausen, 1999). AP2 binding to cargo can only be a part of the internalization process, however, since the binding affinities are weak and the same signals can also be recognized by the AP1 and AP3 adaptors to mediate sorting from Golgi and endosomal membranes.

One additional family of proteins that participates in the endocytotic process are the synaptotagmins, a family of proteins primarily implicated in calcium-regulated exocytosis. Up to 13 synaptotagmin isoforms have been isolated (Matthew et al., 1981; Li et al., 1995; Babity et al., 1997; von Poser et al., 1997; von Poser and Sudhof, 2000; Fukuda and Mikoshiba, 2001). The best studied member of the family is synaptotagmin 1, a neuronal isoform present on the membranes of synaptic vesicles and secretory granules (Matthew et al., 1981). Genetic evidence in *Drosophila*, *C. elegans*, and

mouse have implicated synaptotagmin 1 as an essential component of fast calcium-dependent neurotransmitter release (Littleton et al., 1993; Nonet et al., 1993; DiAntonio and Schwarz, 1994; Geppert et al., 1994; Littleton et al., 1994; Fernandez-Chacon et al., 2001; Littleton et al., 2001). Biochemical properties of synaptotagmin 1 support the idea that synaptotagmin 1 forms part of the calcium sensor in fast exocytosis. Synaptotagmins are integral membrane proteins that contain two calcium binding domains, C2A and C2B, in their large cytoplasmic region (Perin et al., 1991; Sudhof and Rizo, 1996). Calcium triggers binding of the C2A domain to phospholipids (Brose et al., 1992; Davletov and Sudhof, 1993; Chapman and Jahn, 1994; Chapman and Davis, 1998) as well as the clustering of synaptotagmin via the C2B domain (Chapman et al., 1996; Sugita et al., 1996). The two C2 domains cooperate to form complexes with components of the SNARE machinery (Li et al., 1995; Davis et al., 1999; Gerona et al., 2000), suggesting a model in which synaptotagmin triggers exocytosis through its interactions with membranes and the SNARE complex.

A role for synaptotagmin in endocytosis as well as exocytosis was first suggested by the high affinity, calcium-independent binding of the C2B domain to AP2 (Zhang et al., 1994), one of the most conserved properties among the synaptotagmin family (Li et al., 1995). Synaptotagmin 1 binds most tightly to a region of the  $\mu$  chain of AP2 that is

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Key words: synaptotagmin; endocytosis; internalization signal; AP2 adaptor; synaptic vesicle

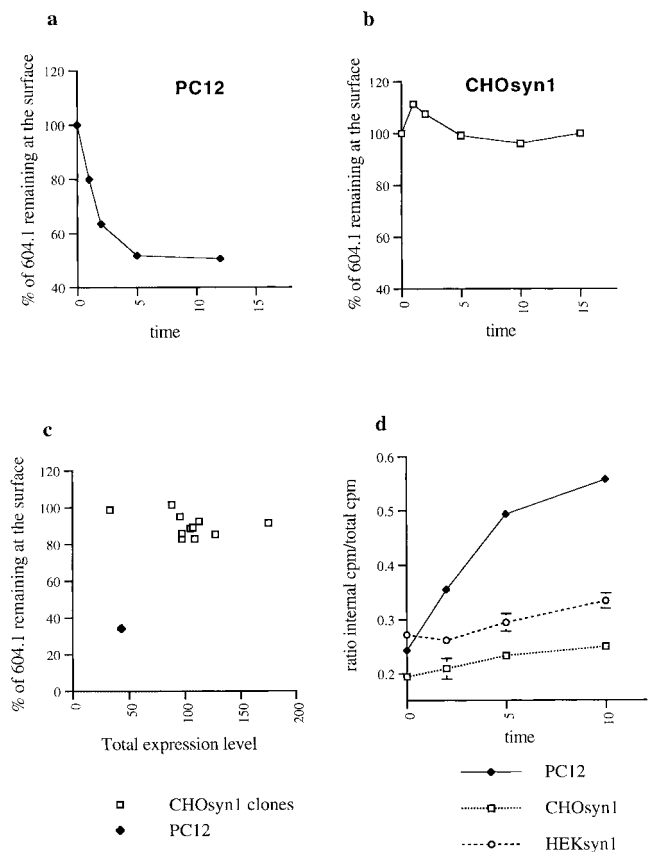
distinct from the AP2 region that binds tyrosine-based internalization signals (Owen and Evans, 1998; Haucke et al., 2000). Transfection experiments have confirmed that the synaptotagmins play a major role in receptor-mediated endocytosis. In one set of experiments, expression of the transmembrane domain of synaptotagmins reduced clathrin-coated pit formation and inhibited LDL uptake (von Poser et al., 2000). This effect was dependent on the presence of two cysteines involved in synaptotagmin oligomerization. In a second set, disrupting the C2B-AP2 interactions *in vivo* by expressing the synprint region of calcium channels inhibited transferrin receptor internalization (Haucke et al., 2000). Moreover, interaction among cargo proteins, AP2, and synaptotagmin appears to be cooperative, since the presence of a peptide containing a tyrosine-based internalization signal enhanced AP2 binding to synaptotagmin *in vitro* (Haucke and De Camilli, 1999). Therefore, synaptotagmins play a critical role in nucleating a coated pit-containing cargo, AP2, and clathrin, even in nonneuronal cells.

The brain-specific synaptotagmin 1 member of the synaptotagmin family appears to exhibit tissue-specific endocytosis, a relatively rare phenomenon. Although synaptotagmin 1 is readily internalized by the neuronal-like PC12 cells, in transfected fibroblasts it was found mostly on the cell surface (Feany et al., 1993). This suggested that cells of the neuroendocrine lineage possess an endocytotic mechanism lacking in nonneuronal cells. To try to understand this mechanism, we have examined the internalization of synaptotagmin 1 and its chimeras into neuronal and nonneuronal cell lines. Surprisingly, the AP2 binding site in the C2B domain is not needed for internalization of chimeras. By contrast, we find evidence for an internalization signal in the COOH-terminal domain of synaptotagmin 1. The internalization signal is sufficient to mediate internalization of chimeras in the absence of the C2B domain, and can be recognized by the endocytotic machinery even in nonneuronal cells. The C2B domain acts as a regulator of internalization since in fibroblast cells it normally conceals the synaptotagmin 1 internalization signal. In PC12 cells, little inhibition of the COOH-terminal internalization signal by the C2B domain is seen unless mutations are made in the C2B domain, in particular in a dilysine motif that has already been implicated in endocytosis by the synprint experiments of Haucke and De Camilli (1999). Endocytosis of synaptotagmin 1 appears to be controlled by a latent internalization signal that is activated by a neuroendocrine-specific mechanism acting via the C2B domain. Thus, synaptotagmin 1 internalization resembles ligand-activated endocytosis of surface receptors, except that the activation is intracellular.

## Results

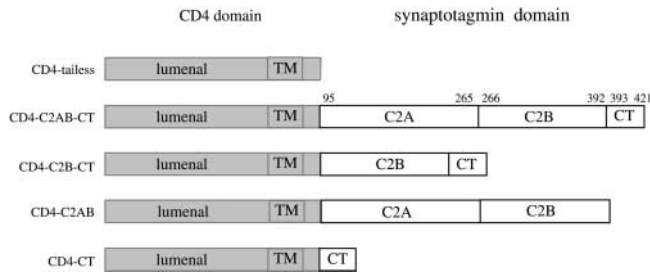
### Synaptotagmin 1 is not endocytosed in CHO cells

In previous studies of CHO cells transfected with synaptic vesicle proteins, synaptophysin, VAMP2, and SV2 were targeted to intracellular organelles (Linstedt and Kelly, 1991; Feany et al., 1993; Grote and Kelly, 1996). In contrast, synaptotagmin 1, when expressed in CHO cells, was detected



**Figure 1. Comparison of synaptotagmin 1 internalization in CHO and PC12 cells.** (a) wtPC12 or (b) CHO stably transfected with synaptotagmin 1 (CHOsyn1) were labeled at 4°C with the 604.1 antibody and then moved to 37°C for the indicated periods. Cells were cooled to 4°C and antibody remaining at the surface after the 37°C incubation was detected with a fluorescein-conjugated secondary antibody. The intensity of fluorescence was determined by flow cytometry. Data were expressed as the percentage of the initial value at  $t = 0$ . (c) The expression level of synaptotagmin 1 in different CHOsyn1 clones was determined by flow cytometry after permeabilization of the cells and staining with 604-1 antibody. These values are expressed along the x-axis. The same clones were then analyzed for internalization of synaptotagmin 1 using the same assay as in panels a and b. The values obtained after 10 min at 37°C correspond to the y-axis. The same measurements were done in parallel on PC12 cells. (d) wt PC12, CHOsyn1, and HEK cells stably expressing synaptotagmin 1 (HEKsyn1) were examined for internalization of synaptotagmin 1 using  $^{125}\text{I}$ -604.1 antibody. Cells were labeled at 4°C and shifted to 37°C for different time points. The internalized antibody was determined by surface acid stripping and expressed as a fraction of total cell associated counts. Each time point was done in triplicate. In this and subsequent figures, when standard deviations are not apparent, they were too small to be represented graphically.

predominantly at the plasma membrane (Feany et al., 1993). To determine whether CHO cells failed to internalize synaptotagmin 1, we generated CHO cells stably transfected with synaptotagmin 1 and developed a quantitative assay of endocytosis using flow cytometry analysis. For this assay, the pool of synaptotagmin 1 on the plasma membrane was labeled at 4°C with the 604.1 antibody directed against the luminal domain of the protein. After warming to 37°C for various times, the 604.1 antibody remaining at the cell



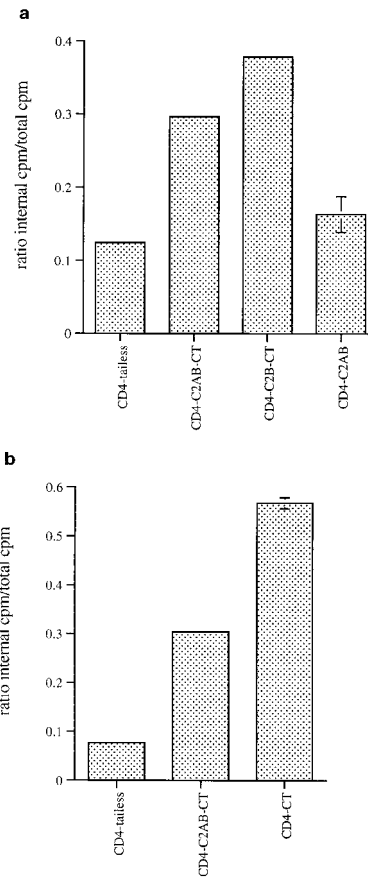
**Figure 2. CD4-synaptotagmin 1 constructs.** A CD4 tailless fragment (human CD4 residues 1–426) was fused to different domains of the cytoplasmic region of rat synaptotagmin 1 to generate the following constructs: CD4-C2AB-CT (synaptotagmin 1 residues 95–421), CD4-C2B-CT (synaptotagmin 1 residues 266–421), CD4-C2AB (synaptotagmin 1 residues 95–392), and CD4-CT (synaptotagmin 1 residues 393–421).

surface was detected by staining with a fluorescent anti-IgG and quantified using flow cytometry. When measured in PC12 cells, internalization of the endogenous synaptotagmin 1 was rapid (Fig. 1 a). About 50% of 604.1 disappeared from the cell surface within the first 5 min. This disappearance was not due to a loss of antibody into the media, since it could be detected inside the cells after permeabilization (data not shown). In contrast, we could not detect significant internalization of synaptotagmin 1 in stably transfected CHO cells (Fig. 1 b), whereas transferrin internalization was normal (data not shown). One possible explanation was that the pathway for synaptotagmin 1 internalization can be saturated at high expression levels. We selected several clones with relatively low expression levels. The total levels of expression were measured by flow cytometry after the staining of permeabilized cells with 604.1 antibody followed by a fluorescent secondary antibody. We examined these same clones in the internalization assay described above. Regardless of the total level of expression, the amount of 604.1 remaining at the surface after 10 min at 37°C was between 75 and 100% of the initial value, even in a clone whose expression level matched that of PC12 (Fig. 1 c). That was in clear contrast with the result obtained in PC12 cells where <40% was detected at the surface after 10 min at 37°C. Our results were confirmed using a conventional endocytosis assay based on internalization of <sup>125</sup>I-604.1 into an acid-resistant pool (Fig. 1 d). Synaptotagmin 1 was not internalized when transfected into CHO cells, or into human embryonic kidney (HEK)\* cells, another nonneuronal cell line. It thus appears as if nonneuronal cells lack some components or pathways involved in synaptotagmin 1 internalization.

### Internalization of synaptotagmin 1 in PC12 cells is mediated by an internalization signal present in the COOH-terminal domain

The lack of internalization of synaptotagmin 1 in CHO cells suggested that synaptotagmin 1 might be internalized by a neuron-specific sorting motif recognized by components

\*Abbreviations used in this paper: HEK, human embryonic kidney; ML, methionine-leucine.



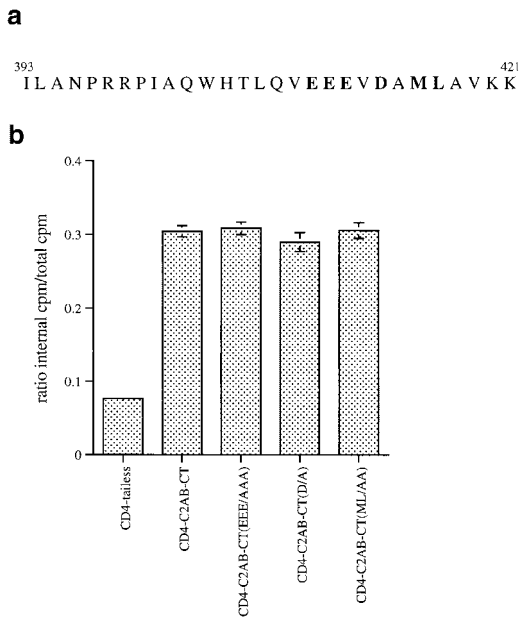
**Figure 3. Internalization of CD4-synaptotagmin 1 constructs in PC12 cells.** Cells stably transfected with retroviral vectors were surface labeled with <sup>125</sup>I-Q4120. Cells were then warmed to 37°C for 10 min and endocytosis was stopped by returning the cells to ice. The internalized <sup>125</sup>I-Q4120 antibody was quantitated as noted in the legend to Fig. 1 d, except that a background level obtained in cells kept at 4°C was subtracted. Error bars represent standard deviation of triplicates in one representative experiment. (a) The COOH-terminal domain of synaptotagmin 1 was necessary for endocytosis of CD4-synaptotagmin 1 constructs. (b) The COOH-terminal was sufficient to promote endocytosis of a CD4-CT construct.

present only in neurons. Since the C2B domain is known to bind AP2 it might contain the internalization signal. The AP2 binding site in the C2B domain has been mapped in a region between residues 296 and 328 (Chapman et al., 1998). To identify the cytoplasmic domain responsible for internalization of synaptotagmin 1 in PC12 cells, we generated constructs containing the luminal and transmembrane domain of the CD4 molecule fused to different cytoplasmic regions of synaptotagmin 1 (Fig. 2). Each construct was stably expressed in PC12 cells by retroviral infection and tested for internalization using uptake of <sup>125</sup>I-Q4120, a well characterized antibody directed against the external part of CD4. Cell surface CD4 was labeled at 4°C and the antibody was allowed to internalize at 37°C for 10 min. Internalization of <sup>125</sup>I-Q4120 was assessed as acid-resistant bound antibody. As expected from previous studies (Pelchen-Matthews et al., 1991), a CD4 tailless construct was only poorly endocytosed (Fig. 3 a). Its internalization was efficiently promoted by fusion to the cytoplasmic domain of synaptotagmin 1 contain-

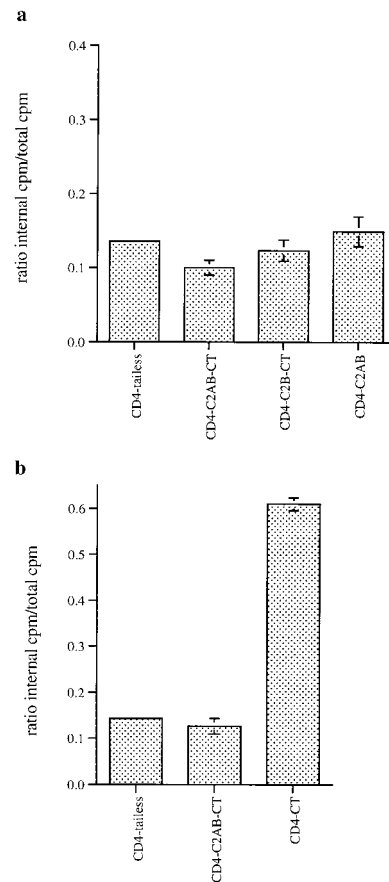
ing the two C2 domains and the COOH-terminal region (CD4-C2AB-CT).

The difference in the endocytotic rates in the presence and absence of the cytoplasmic tail of synaptotagmin 1 allowed us to initiate a mutagenesis study. Deleting the C2A domain did not inhibit internalization (CD4-C2B-CT), but surprisingly the C2B domain could not promote internalization signal when the COOH-terminal region was removed (CD4-C2AB), although the AP-2 binding site was intact. Removal of the COOH-terminal region (CD4-C2AB) reduced internalization to a level close to that of the CD4 tailless construct. In contrast, the COOH-terminal region alone, in the absence of C2A and C2B domains, was sufficient to promote internalization of a CD4-CT construct (Fig. 3 b). Kinetic analysis of internalization of the mutants confirmed that the steady state measurements accurately reflect changes in the initial rates of internalization. We can thus conclude that the AP2 binding site is not essential for internalization of synaptotagmin 1. Instead, a strong internalization motif is present in the COOH-terminal domain of synaptotagmin 1 that is necessary and sufficient for internalization.

We then examined whether the motifs that conformed to known internalization signals in the COOH-terminal domain were responsible for synaptotagmin 1 internalization. At present, two major groups of internalization motifs have



**Figure 4. Mutagenesis of the putative internalization motifs within the COOH-terminal domain of synaptotagmin 1.** (a) The 29 amino acid sequence of the COOH-terminal domain of synaptotagmin 1 is shown. The cluster of acidic residues, E410, 411, 412, and D414, as well as the synaptic vesicle targeting signal, M416, L417, are indicated in bold type. (b) Changes to alanine were made in the CD4-C2AB-CT construct as follows: E410, 411, 412A to generate CD4-C2AB-CT(EEE/AAA), D414A to generate CD4-C2AB-CT(D/A), and M416A, L417A to generate CD4-C2AB-CT(ML/AA). Internalization of the constructs was assayed as in Fig. 3.



**Figure 5. Internalization of CD4-synaptotagmin 1 constructs in CHO cells.** Cells stably transduced with retroviral vectors were analyzed for internalization of  $^{125}\text{I}$ -Q4120 antibody as in Fig. 3. (a) The C2AB-CT, C2B-CT, or C2AB domain of synaptotagmin 1 did not promote internalization of CD4-synaptotagmin 1 chimeras. (b) The COOH-terminal domain of synaptotagmin 1 did promote internalization when fused alone to the CD4 tailless molecule.

been described (Mellman, 1996; Kirchhausen et al., 1997), tyrosine-based motifs and dileucine-based motifs in which one of the leucines can be substituted by isoleucine, methionine, or valine (Bremnes et al., 1994). The COOH-terminal domain in our CD4-CT is composed of 29 amino acids, starting with an isoleucine-leucine motif (synaptotagmin 1 residues 393, 394; Fig. 4 a). In a study of synaptic vesicle targeting signals (Blagoveshchenskaya et al., 1999), internalization of a CD4-CT construct lacking the isoleucine-leucine motif was observed, showing that the motif is unlikely to be part of the internalization signal. The COOH-terminal domain contains a methionine-leucine (ML) motif (synaptotagmin 1 residues 416, 417) that was shown to mediate part of the synaptotagmin 1 targeting to synaptic-like microvesicles (Blagoveshchenskaya et al., 1999). The authors reported that this motif was not acting at the step of synaptotagmin 1 internalization. The ML motif is preceded by a cluster of acidic residues. Acidic residues have been shown to be involved in targeting events, either by themselves or in combination with tyrosine or dileucine-based motifs (Matter et al., 1994; Pond et al., 1995; Voorhees et al., 1995; Piguet et al., 2000). To assess the function of these putative

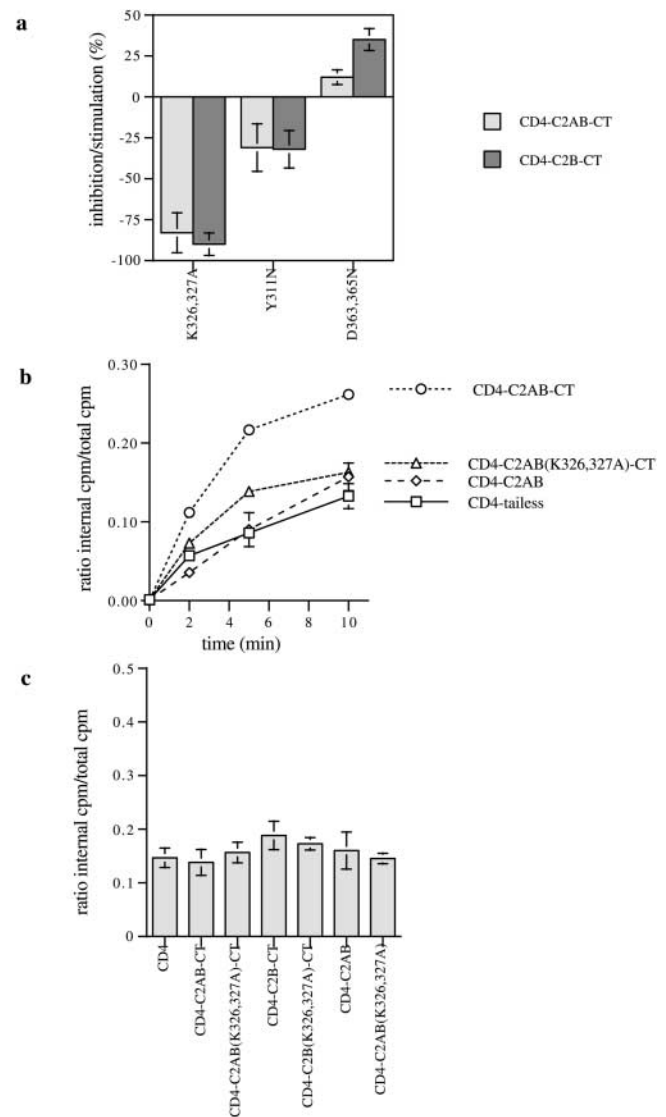
internalization signals, we substituted them for alanine residues in our CD4-C2AB-CT construct. Mutation of one (D414A) or three (E410,411,412A) of the acidic residues did not affect internalization of the CD4-C2AB-CT construct (Fig. 4 b). Simultaneous mutation of M416A, L417A also had no effect on internalization of the CD4-C2AB-CT construct, thus confirming that the synaptic vesicle targeting signal was not affecting synaptotagmin 1 internalization. These results suggest the existence of an unconventional internalization signal in the COOH-terminal region of synaptotagmin 1.

### The C2B domain inhibits internalization in CHO cells

Synaptotagmin 1 was not internalized in CHO cells. Once the COOH-terminal region was known to contain the internalization signal, we could test the hypothesis that it is specific for neuronal cells. To do this, the CD4-synaptotagmin constructs were stably expressed in CHO cells using the same retroviral system as the one used to transduce PC12 cells. Consistent with our previous observations showing that synaptotagmin 1 was not endocytosed in CHO cells, the CD4-C2AB-CT construct was not internalized (Fig. 5 a). Removal of the C2A or the CT domains did not affect the retention of the constructs at the surface. In contrast, the CD4-CT construct was very efficiently internalized, at levels comparable to that obtained in PC12 cells (Fig. 5 b). This result clearly indicates that the internalization signal present in the COOH-terminal domain is efficiently recognized in CHO cells, ruling out the hypothesis of a neuron-specific internalization signal. Addition of the C2B domain was sufficient to inhibit the function of the COOH-terminal internalization signal in CHO cells, since the CD4-C2B-CT construct was not internalized (Fig. 5 a). This implied that the C2B domain was a regulator of endocytosis, inhibiting the internalization signal in CHO cells, but not in PC12 cells.

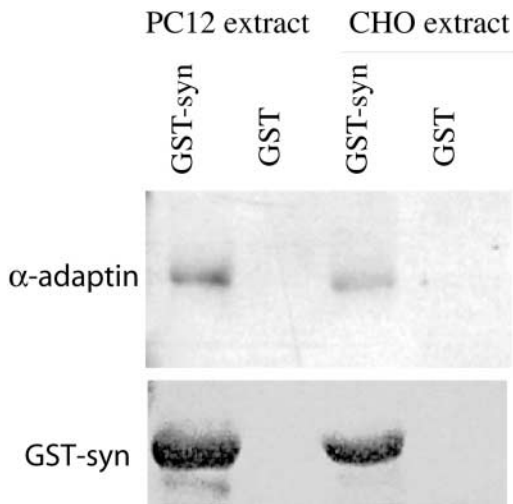
### Mutations within the C2B domain affect endocytosis of synaptotagmin 1 in PC12 cells

Although the C2B domain is not needed for internalization, we have found that mutations within it modify the internalization process in PC12 cells, consistent with a role as a regulator. We have analyzed three sets of mutations in the C2B domain by taking advantage of previous studies that examined oligomerization of synaptotagmin or its role in exocytosis. The K326,327A mutation is known to affect several C2B-mediated interactions, in particular AP2 binding and calcium-mediated dimerization (Chapman et al., 1998). The Y311N mutation was reported to inhibit calcium-mediated conformational changes in the C2B and to reduce calcium-mediated dimerization (Littleton et al., 2001). It lies in close proximity to a set of conserved acidic calcium ligands within the C2B domain (D303, 309, 363, 365, 371) (Desai et al., 2000; Littleton et al., 2001). The Y311N mutation corresponds to the AD3 mutation in *Drosophila* that results in a postdocking defect in vivo. The D363,365N mutation, which is believed to mimic the charge neutralization induced by calcium binding, has been reported to promote constitutive dimerization of synaptotagmin 1 (Desai et



**Figure 6. Effect of the K326,327A, Y311N, and D363,365N mutations on internalization of CD4-synaptotagmin 1 chimeras.** (a) PC12 transduced with retroviral vectors were assayed as in Fig. 3, and a ratio (R) of internal cpm divided by total cpm was calculated as before. The extent of inhibition (negative values) or stimulation (positive values) was calculated for each mutation as follows:  $R_{\text{mutation-Rwt}}/R_{\text{wt-RCD4 tailless}}$ . Results in panel a, represent the average of three independent experiments. The K326,327A mutation dramatically affected internalization of the CD4-synaptotagmin 1 constructs in PC12 cells. (b) PC12 transduced with retroviral vectors were assayed as in Fig. 3, except that cells were left at 37°C for different periods of time. (c) CHO cells stably transduced were assayed as in Fig. 3. The K326,327A mutation had no effect in CHO cells.

al., 2000). We introduced these mutations in the CD4/synaptotagmin 1 constructs and tested the ability of the mutants to be internalized in PC12 cells. We found that the K326,327A mutation in the C2B domain dramatically reduced the extent of internalization (Fig. 6 a), even though the domain is not required for internalization (Fig. 3 b). This effect was seen in two independent constructs, CD4-C2AB(K326/327A)-CT and CD4-C2B(K326/327A)-CT. The inhibitory effect of the K326,327A mutation was confirmed in a kinetic experiment (Fig. 6 b). The mutation had



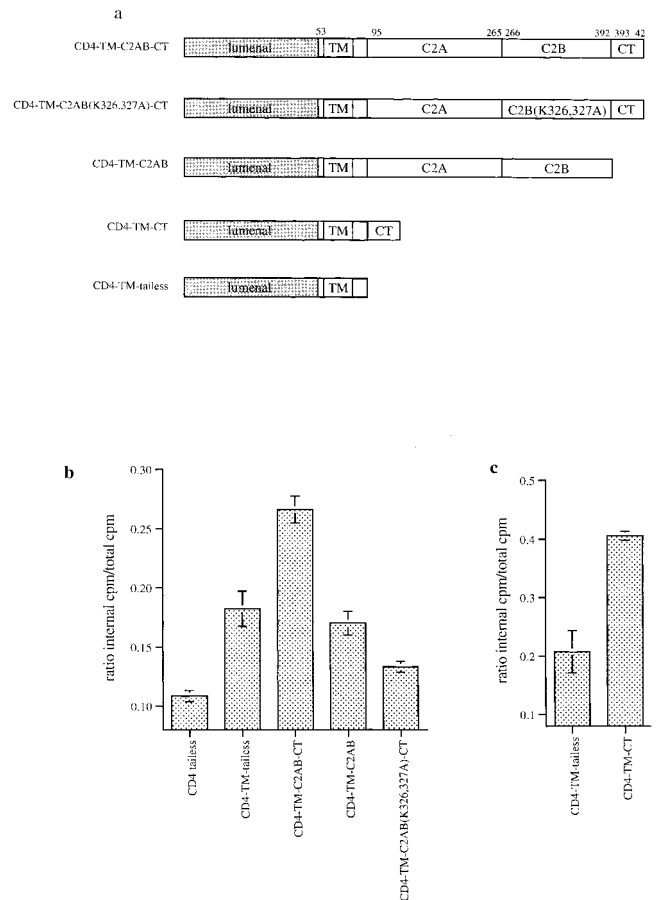
**Figure 7. AP2 binding to synaptotagmin 1 in PC12 and CHO cells.** Cell extracts were incubated with glutathione-agarose beads bound to GST or GST fused to the cytoplasmic domain of synaptotagmin 1 (GST-syn). Material bound to the bead was eluted, separated by SDS-PAGE electrophoresis, and analyzed by Western blot with an anti  $\alpha$ -adaptin antibody (top). The bottom shows the GST-syn protein as visible after Ponceau staining.

no effect in CHO cells (Fig. 6 c), showing that the C2B region giving negative regulation in CHO cells is separate from its AP2 binding site. The Y311N mutation had a partial inhibitory effect on synaptotagmin 1 internalization in PC12 cells (Fig. 6 a). The average extent of inhibition was  $\sim$ 30%, whereas the K326,327A mutation inhibited to  $>$ 80% (average calculated on three independent experiments). Conversely, we observed a slight stimulation of internalization when the D363,365N mutation was present in the CD4-C2B-CT construct and no effect within the context of the CD4-C2AB-CT chimera (Fig. 6 a).

A GST/C2AB-CT fusion protein pulled down AP2 from both PC12 and CHO cytosol (Fig. 7), indicating that the different internalization properties of CHO and PC12 cells are not due to a CHO-specific factor that prevents AP2 binding.

### The internalization signal as well as the regulatory domain of synaptotagmin 1 function independently from the transmembrane region

Overexpression of the transmembrane domain of synaptotagmins inhibits LDL receptor endocytosis (von Poser et al., 2000). Although the effect was shown to be dependent on two cysteines involved in synaptotagmin oligomerization, the precise mechanism of the inhibitory effect is unknown. This study suggested that the oligomerization via the transmembrane region of synaptotagmins was important for the assembly of clathrin-coated pits in nonneuronal cells. Wild-type synaptotagmin 1 showed no detectable endocytosis into transfected CHO cells, whereas it was efficiently internalized in PC12 cells (Fig. 1). Similar results were found using CD4/synaptotagmin 1 constructs lacking the transmembrane domain of synaptotagmin 1 (Figs. 3 a and 5 a). Thus, the transmembrane domain can-



**Figure 8. CD4-synaptotagmin 1 constructs containing the transmembrane domain of synaptotagmin 1.** (a) The CD4 luminal domain was fused to the transmembrane and cytoplasmic regions of synaptotagmin 1 to generate CD4-TM-C2AB-CT (synaptotagmin 1 residues 53–421), CD4-TM-C2AB(K326,327A)-CT (same as CD4-TM-C2AB-CT but with the presence of the K326,327A mutation), CD4-C2AB (synaptotagmin 1 residues 53–393) and CD4-TM-CT (synaptotagmin 1 residues 53–94 and 393–421). As a control, a CD4-TM tailless construct (synaptotagmin 1 residues 53–94) was generated by introduction of a stop codon after residue 94 of synaptotagmin 1; this construct contains the transmembrane domain of synaptotagmin 1 followed by the short proximal cytoplasmic domain. (b and c) PC12 transduced with retroviral vectors were assayed as in Fig. 3. The effects of the CT region and of the K326,327A mutation are independent of the presence of the transmembrane domain.

not be important for the tissue-specific internalization of the protein. To ask if recognition of the COOH-terminal internalization signal was influenced by the nature of the transmembrane domain in PC12 cells, constructs were made that contained the luminal domain of CD4 fused to the transmembrane domain of synaptotagmin 1 and its different cytoplasmic regions (Fig. 8 a). We found again that the COOH-terminal region was necessary and sufficient to promote efficient internalization of the chimeras in PC12 cells (Fig. 8, b and c), whereas the K326,327A mutation completely abolished internalization (Fig. 8 b). Thus, the function of the COOH-terminal region as an internalization signal, as well as the C2B domain as a regulatory re-

gion, is independent of the nature of the transmembrane domain.

## Discussion

This investigation was stimulated by puzzling observations about the synaptotagmins. Synaptotagmin 1 did not get targeted to internal compartments like other synaptic vesicle proteins when transfected into fibroblasts, but instead accumulated on the cell surface (Feany et al., 1993). We thought that this might be due to an overexpression artifact that saturated the endocytotic pathway in transfected cells, but this was not so (Fig. 1 c). This rare example of cell type-specific endocytosis suggested the involvement of neuroendocrine-specific factors required for internalization in cells with regulated secretory pathways. It makes sense that regulated secretory cells might have a specialized endocytotic machinery to handle compensatory endocytosis, which is the recovery of secretory vesicle membranes after calcium-mediated exocytosis (Jarousse and Kelly, 2001).

Our data suggest that the tissue specificity of synaptotagmin 1 endocytosis is due to a regulatory mechanism that is only activated in PC12 cells. Although synaptotagmins bind AP2 (Zhang et al., 1994; Li et al., 1995), AP2 by itself cannot explain the neuroendocrine specificity that we observe. Indeed, AP2 is present in the cytosols of both fibroblasts and PC12 cells and can bind to immobilized synaptotagmin tails (Fig. 7). Moreover, the AP2 binding site does not act as an internalization motif since its deletion did not inhibit internalization in PC12 cells (Fig. 3 b). Instead, the internalization signal was found in the COOH-terminal region of the protein (Fig. 3). In absence of the C2B domain, the COOH-terminus internalization signal could promote endocytosis in both PC12 and CHO cells (Figs. 3 b and 5 b). Thus, the tissue specificity of synaptotagmin 1 internalization is not due to a neuron-specific internalization motif, but to differences in the accessibility of the internalization signal. This accessibility appears to be controlled by the C2B domain, which totally inhibits internalization in CHO cells (Fig. 5 a) and also in PC12 cells when the oligomerization domain is inactivated by the K326,327A mutation (Fig. 6). Internalization in PC12 cells is also influenced, albeit more weakly, by the calcium binding region of the C2B domain. A mutation that inhibited calcium-mediated conformational changes (Y311N) reduced endocytosis, while one that partially neutralizes the charge (D363,365N) enhanced it (Fig. 6 a). The D363,365N was reported to cause the C2B domain to dimerize as if it had calcium bound (Desai et al., 2000).

C2 domains are usually thought to regulate membrane traffic by their ability to sense changes in intracellular environment, often with calcium levels, then trigger association with membranes. Synaptic vesicle recycling has been shown to be crucially dependent on the presence of synaptotagmin (Jorgensen et al., 1995), particularly its C2B domain (Fukuda et al., 1995b; Littleton et al., 2001). We propose that the C2B domain acts as a sensor of intracellular changes in neurons or neuroendocrine cells and couples these changes to endocytosis by activating a novel internalization signal. A plausible change that the C2B domain might sense is that a calcium-mediated exocytotic event has occurred. The C2B

domain could detect such an event in several ways. The K326,327 motif has been implicated in calcium-dependent dimerization of synaptotagmin 1 (Chapman et al., 1998), which in turn is thought to be required for exocytosis (Desai et al., 2000). If calcium-mediated dimerization were also to expose the COOH-terminal internalization domain, then endocytosis would be elegantly coupled to exocytosis. Calcium-mediated dimerization of synaptotagmin 1 cannot fully explain the regulation of its endocytosis, however, since the D363,365N mutation, which gives constitutive dimerization (Desai et al., 2000), did not restore internalization in CHO cells (data not shown). An alternative to calcium-mediated dimerization activating internalization of synaptotagmin 1 is binding via its dilysine motif to calcium channels (Chapman et al., 1998), to phosphorylated phosphatidyl inositides (Fukuda et al., 1995a), or to an unknown protein on the plasma membrane. If any such interaction activates the internalization signal, synaptotagmin 1 would only bind coats after its arrival at the cell surface, not while a component of an intracellular organelle. Since AP2 also binds to the dilysine motif, an unattractive feature of these conjectures is that they require the absence of AP2 in order to activate a coating step. AP2 binding could still play a significant role if the neuroendocrine-specific factor activated endocytosis when it interacted with AP2 bound to the C2B domain. Candidates for such AP2 binding factors abound and include AP180 and the nerve-specific forms of endophilin, syndapin, dynamin, and intersectin. Several of these factors have been shown to cluster at "hot spots" on the plasma membrane of nerve terminals (Estes et al., 1996; Gonzalez-Gaitan and Jackle, 1997; Roos and Kelly, 1998) that are sometimes adjacent to sites of exocytosis (Roos and Kelly, 1999). If they released an inhibition, this would provide a mechanism for restricting synaptotagmin 1 internalization to these specialized endocytotic zones. However, in preliminary experiments, cotransfection of CHO cells with synaptotagmin and AP180 or dynamin 1 has not activated the internalization of synaptotagmin (data not shown). However, an AP2 binding protein need not be cytoplasmic. A complex of AP2 with a synaptic vesicle cargo protein could trigger internalization of synaptotagmin 1.

An earlier study suggested that oligomerization of synaptotagmin via its transmembrane domain plays a role in formation of clathrin-coated pits (von Poser et al., 2000). Since our first series of constructs (Fig. 2) lacked this domain, we generated a second series of chimeras (Fig. 8 a) to verify that our observations were still true in the presence of the transmembrane domain of synaptotagmin 1. Our results clearly show that the COOH-terminal region acts as an internalization signal even in presence of the transmembrane domain, and that the K326,327A mutation abolishes internalization. A puzzling observation, though, is that the tailless construct containing the synaptotagmin transmembrane domain was internalized to a higher extent than the one containing the CD4 transmembrane region (CD4-TM tailless and CD4 tailless, respectively; Fig. 8 b). A possible mechanism is that the synaptotagmin transmembrane region forms oligomers (von Poser et al., 2000) with the endogenous synaptotagmin in PC12 cells, thus resulting in internalization of the CD4-TM tailless via a piggy back mechanism. Alternatively, the

transmembrane and/or the short proximal cytoplasmic domain (to residue 94) may contain an additional weak internalization signal. Both transmembrane and COOH-terminal signals are inhibited by the K326,327A mutation (Fig. 8 b).

Important questions remain about the role of the synaptotagmins in nonneuronal cells. The COOH-terminal internalization signal of synaptotagmin 1 is recognized in fibroblasts by a machinery that cannot be cell type-specific. A peptide that blocks the binding of AP2 to the C2B domain of an endogenous synaptotagmin inhibited transferrin uptake in fibroblasts (Haucke et al., 2000). One possible interpretation is that the peptide used in this study inhibited an activating region required for exposure of an internalization signal. The synaptotagmins are now known to regulate exocytosis in neuronal cells as well as nonneuronal ones (Martinez et al., 2000). Our data hint that they might also be widespread regulators of endocytosis.

Our analysis reveals an unexpected complexity in synaptotagmin 1 internalization, which is controlled by a mixture of internalization and regulatory signals to give tissue-specific internalization. Another example of tissue-specific internalization was reported for a specific isoform of the Fc receptor family, FcRII-B1. In this case, a B cell-specific in frame insertion into an internalization signal prevented the lymphocytes from executing FcR-dependent antigen presentation (Miettinen et al., 1989; Amigorena et al., 1992). Exposure of latent endocytotic signals has been reported for receptor tyrosine kinases or G protein-coupled receptors that undergo ligand-stimulated endocytosis (Ferguson, 2001). The presence of a latent signal in synaptotagmin 1 and the involvement of the synaptotagmins in both compensatory endocytosis in the nerve terminals (Jorgensen et al., 1995) and constitutive endocytosis in fibroblasts (Haucke et al., 2000; von Poser et al., 2000) implies that these other forms of endocytosis might also be regulated, perhaps via the C2B domain of synaptotagmins. In contrast to ligand-stimulated endocytosis, however, the signals controlling endocytosis rates are intracellular.

## 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. HEK cells and Phoenix cells (American Type Culture Collection) were grown in DMEH-21 media supplemented with 10% FCS and penicillin-streptomycin.

### Reagents and antibodies

The cDNA for rat synaptotagmin 1 was provided by Dr. Kathleen M. Buckley (Harvard Medical School, Boston, MA). The pGEX-synaptotagmin 1 plasmid encoding the GST protein fused to the cytoplasmic region of rat synaptotagmin 1 was a gift from Dr. Thomas C. Südhof (University of Texas Southwestern Medical Center, Dallas, TX). The pcDNA3-CD4 and pBMN-Z-I Neo plasmids were provided by Dr. Don Ganem (University of California at San Francisco, San Francisco, CA).

Monoclonal antibody against the luminal domain of synaptotagmin 1 (clone 604.1) was provided by Dr. R. Jahn (Goettingen, Germany). Monoclonal antibody against the external domain of the human CD4 (clone Q4120) was obtained from the Medical Research Council AIDS Reagents Program (National Institute for Biological Standards and Control). Anti- $\alpha$ -adaptin antibody was purchased from Transduction Laboratories.

### Constructs

The rat synaptotagmin 1 ORF was subcloned in the pcDNA3-hygro vector (Invitrogen).

For the CD4-synaptotagmin constructs, a CD4 fragment (corresponding to residues 1–426) encoding the luminal, transmembrane, and 12 amino acids of the cytoplasmic region of the human CD4 was amplified by PCR from pcDNA3-CD4. The primers were chosen so that a SmaI restriction site followed by a stop codon was added to the 3' end of the CD4 fragment. The PCR product was subcloned in the pCR3.1 vector (T/A cloning kit; Stratagene), resulting in the pCR3.1-CD4 tailless plasmid. The cytoplasmic domains of synaptotagmin 1 were amplified by PCR from the pGEX-synaptotagmin 1 plasmid to generate the following fragments: C2AB-CT (encoding for residues 95–421), C2AB (residues 95–392), C2B-CT (residues 266–421), and CT (residues 393–421). The forward primers were flanked with a SmaI restriction site. The PCR products were subcloned in the pCR3.1 vector. The synaptotagmin 1-encoding regions were then cut out as SmaI-XhoI fragments and ligated to the corresponding sites of the pCR3.1-CD4 tailless to generate in-frame CD4/synaptotagmin 1 chimeras. The resultant constructs were sequenced and the CD4/synaptotagmin 1 ORFs were introduced as BamHI-XhoI fragments in the pBMN-Z-I-Neo-derived vector. The K326,327A mutants were generated the same way, except that the synaptotagmin 1 fragments were amplified from pGEX-synaptotagmin 1-KK/AA plasmid, in which the K326,327A had been introduced using a QuikChange site-directed mutagenesis kit (Stratagene). The other point mutations in the CD4-C2AB-CT and CD4-C2B-CT constructs were generated by PCR using standard procedures. A synaptotagmin 1 fragment containing the transmembrane and proximal cytoplasmic domain (corresponding to amino acids 53–94) was amplified by PCR from the pcDNA3-synaptotagmin 1 vector and fused in frame by PCR to the luminal domain of the CD4 molecule (CD4 residues 1–394). The resulting chimeric fragment was flanked with a BamHI and a SmaI site at the 5' and 3' ends, respectively. The PCR product was subcloned as a BamHI-SmaI fragment in the pCR3.1 plasmids either upstream of a stop codon to generate the CD4-TM tailless construct (synaptotagmin 1 residues 53–94), or upstream of the synaptotagmin 1 cytoplasmic domains to give the following constructs: CD4-TM-C2AB-CT (synaptotagmin 1 residues 53–421), CD4-TM-C2AB (synaptotagmin 1 residues 53–392) and CD4-TM-CT (synaptotagmin 1 residues 53–94 and 393–421). The SmaI restriction site used to generate the TM-containing constructs resulted in an insertion of two amino acids (P, G) between amino acid 94 and 95 of synaptotagmin 1. The constructs were sequenced and a subsequent subcloning in the pBMN-Z-I-Neo-derived vector was done as described above.

### Transfections and retroviral infections

CHO and HEK cells were stably transfected with the pcDNA3-synaptotagmin 1 vector using the Fugene-6 product according to the manufacturer's instructions (Boehringer). Cells were selected in the presence of 400  $\mu$ g/ml of hygromycin.

In initial experiments we tried to use transient transfectants to identify sorting motifs. However, FACS analysis revealed an extremely high variability in the level of expression from cell to cell. Since our assays measure the behavior of the transfected protein, they are heavily biased towards cells with high expression levels. Since we were concerned about saturation of sorting pathways in overexpressing cells, we chose instead the route of retroviral infection that gives rise to moderate and homogenous level of expression. For retroviral infections, we used a vector derived from pBMN-Z-I-Neo in which the LacZ gene was deleted (fragments BamHI-Sall) and replaced by our genes of interest (fragment BamHI-XhoI containing the CD4/synaptotagmin 1 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, resulting in nearly all surviving colonies stably expressing 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). 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 and CHO cells. The Phoenix cells were transfected with the different CD4-synaptotagmin 1 constructs using the Fugene-6 product. Virus-containing supernatants were filtered through a low binding protein 0.45- $\mu$ m filter (Pall Corporation) 48 h posttransfection, diluted with 4  $\mu$ g/ml of hexadimethrine bromide (Sigma-Aldrich tailless), and used to infect PC12 or CHO cells. After 12 h, the inoculum was replaced by fresh media; 24–36 h later, 400  $\mu$ g/ml of G418 was added. 10 d after infection, colonies were pooled and propagated in culture in the presence of 400  $\mu$ g/



ml of G418. Cells were treated for 20 h before the experiments with 500 nM of trichostatin A to enhance expression of the constructs.

### Flow cytometry analysis

For internalization assays, cells were detached by using enzyme-free/PBS-based cell dissociation buffer (GIBCO BRL) according to the manufacturer's instructions. Cells were incubated with 2 µg/ml of 604.1 antibody in a saline solution (119 mM NaCl, 2.5 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 25 mM Hepes, pH 7.4, and 30 mM glucose) supplemented with 3% BSA for 60 min at 4°C. Cells were washed, incubated at 37°C for different periods of time, and returned to 4°C to stop endocytosis. The 604.1 antibody remaining at the surface was revealed by incubation at 4°C with a fluorescein-conjugated anti-mouse antibody. Cells were analyzed with a Becton Dickinson FACScalibur. The data were collected in a logarithmic mode, and the geometric mean of fluorescence intensity was calculated.

To measure the expression level of synaptotagmin 1, cells were dissociated in enzyme-free buffer and fixed in 4% paraformaldehyde in PBS. Antibody incubations and washings were performed in PBS 1% BSA in the presence of 0.02% saponin (Sigma-Aldrich). Cells were washed and stained with 604.1 antibody. After washing, bound antibody was visualized by addition of phycoerythrin-conjugated goat anti-mouse antibody. Cells incubated with the secondary antibody alone served as negative controls. Cells were analyzed by flow cytometry as mentioned above.

### Internalization assays using iodinated antibodies

100 µg of 604.1 or 50 µg of Q4120 antibody was iodinated on iodogen-coated tubes (Pierce Chemical Co.) as described (Clift-O'Grady et al., 1998). Cells were plated on collagen and poly-D-lysine-coated 12-well plates 2 d before the assay. Cells were incubated for 1 h at 4°C with 2 µg/ml of <sup>125</sup>I-604.1 antibody or 100 ng/ml of <sup>125</sup>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 to allow endocytosis and then returned to 4°C. Antibody remaining at the cell surface was removed by two 10-min 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 wash-resistant radioactive cpm by the sum of acid wash-resistant and -accessible cpm. A background of acid-resistant counts in cells kept at 4°C was subtracted from each value.

### GST fusion protein pull down assay

The pGEX-synaptotagmin 1 plasmid was used to express the fusion protein according to standard methods. GST control protein was generated by expression of the plasmid pGEX-3X (Amersham Pharmacia Biotech). GST fusion proteins were bound to glutathione-agarose chromatography and subjected to an extensive wash with intracellular buffer (38 mM potassium aspartate, 20 mM potassium MOPS, pH 7.2, 5 mM reduced glutathione, 5 mM sodium carbonate, 2.5 mM magnesium sulfate). The washed beads were used in a standard GST fusion protein binding assay. In brief, PC12 or CHO cell extracts were obtained by lysis with 1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, in the presence of proteases inhibitors. 40 µl of saturated beads were incubated with 500 µg of cell extracts overnight at 4°C. Beads were washed extensively in the Triton X-100-containing buffer and eluted with 20 mM glutathione in 120 mM NaCl, 50 mM Tris, pH 8, for 30 min at room temperature. Proteins were then separated by SDS-PAGE and assayed by Western blotting with an anti-α-adaptin antibody used at 1:1,000.

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