

# Endocytosis of VAMP Is Facilitated by a Synaptic Vesicle Targeting Signal

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**Abstract.** After synaptic vesicles fuse with the plasma membrane and release their contents, vesicle membrane proteins recycle by endocytosis and are targeted to newly formed synaptic vesicles. The membrane traffic of an epitope-tagged form of VAMP-2 (VAMP-TAG) was observed in transfected cells to identify sequence requirements for recycling of a synaptic vesicle membrane protein. In the neuroendocrine PC12 cell line VAMP-TAG is found not only in synaptic vesicles, but also in endosomes and on the plasma membrane. Endocytosis of VAMP-TAG is a rapid and saturable process. At high expression levels VAMP-TAG accumulates at the cell surface. Rapid endocytosis of

VAMP-TAG also occurs in transfected CHO cells and is therefore independent of other synaptic proteins. The majority of the measured endocytosis is not directly into synaptic vesicles since mutations in VAMP-TAG that enhance synaptic vesicle targeting did not affect endocytosis. Nonetheless, mutations that inhibited synaptic vesicle targeting, in particular replacement of methionine-46 by alanine, inhibited endocytosis by 85% in PC12 cells and by 35% in CHO cells. These results demonstrate that the synaptic vesicle targeting signal is also used for endocytosis and can be recognized in cells lacking synaptic vesicles.

**S**IGNALS in the cytoplasmic domains of membrane proteins are often required for endocytosis. The most common type of endocytosis signal contains an essential tyrosine exposed by a type 1  $\beta$ -turn and promotes internalization by interacting with the AP2 adaptor proteins of clathrin-coated pits (reviewed in Trowbridge et al., 1993). Another class of signals that can promote endocytosis has a dileucine motif (Letourneur and Klausner, 1992).

Membrane proteins also often contain additional signals adjacent to or overlapping their endocytosis signals that direct them to other destinations on their membrane trafficking itineraries. We have recently reported the identification of a signal for targeting an epitope-tagged form of VAMP-2 (VAMP-TAG)<sup>1</sup> to synaptic vesicles in transfected PC12 cells (Grote et al., 1995). This synaptic vesicle targeting signal (SVTS) requires hydrophobic amino acids within a predicted amphipathic helix. Point mutations within the SVTS can either increase or decrease the fraction of VAMP-TAG sorted to synaptic vesicles. No signals

homologous to the SVTS are found in other synaptic vesicle membrane proteins, so there cannot be a common sorting receptor analogous to the coated pit adaptors that is responsible for synaptic vesicle targeting. As an alternative, we suggested that VAMP-TAG is targeted to synaptic vesicles by associating, via the SVTS, with other synaptic vesicle membrane proteins.

VAMP-2, also known as synaptobrevin-2, is an 18-kD COOH-terminal anchored synaptic vesicle protein with a critical role in vesicle fusion (Trimble, 1993). Cleavage of VAMP-2 by the metalloprotease subunit of the tetanus neurotoxin blocks synaptic vesicle exocytosis (Schiavo et al., 1992). VAMP-2 is one component of a trimeric complex known as a SNAP receptor, or SNARE, because it serves as a binding site for  $\alpha$ -SNAP and NSF, two components of the intracellular fusion machinery (Sollner et al., 1993). Since VAMP-2 is a synaptic vesicle protein while the other components of the SNARE complex, syntaxin and SNAP-25, are primarily located on the plasma membrane, the SNARE complex may bridge the short gap between a docked synaptic vesicle and the plasma membrane before the fusion event. There is a growing family of proteins homologous to VAMP-2 that are targeted to distinct populations of transport vesicles and are collectively referred to as v-SNAREs. The v-SNAREs on each class of transport vesicle form SNARE complexes with specific t-SNAREs on their unique targets for membrane fusion. Thus, SNARE complex formation may play a role in regulating the specificity of membrane fusion. GTP binding

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1. *Abbreviations used in this paper:* pIgR, polymeric immunoglobulin receptor; SVTS, synaptic vesicle targeting signal; VAMP-TAG, SV-40 T antigen epitope-tagged VAMP-2.

regulatory proteins in the rab family, which are also targeted to distinct populations of transport vesicles and thought to regulate the specificity of membrane fusion, are required *in vivo* for v-SNARE activation and SNARE complex formation (Lian et al., 1994; Sogaard et al., 1994).

The domain of VAMP-2 that is most highly conserved between species, among VAMP isoforms, and with other v-SNAREs is located in the cytoplasm adjacent to the transmembrane anchor. This domain is required for both synaptic vesicle targeting and t-SNARE binding (Hayashi et al., 1994; Grote et al., 1995). However, the sequences required for these two functions can be distinguished by mutations that block or enhance synaptic vesicle targeting but not t-SNARE binding (Grote et al., 1995). The juxtamembrane domain of cellubrevin, a v-SNARE for recycling endosomes, is 96% identical to the analogous domain of VAMP-2 (McMahon et al., 1993). Although cellubrevin is found in many cell types, it is targeted to synaptic vesicles when expressed in neuroendocrine cells (Chilcote et al., 1995; Grote et al., 1995). The promiscuous targeting of cellubrevin implies that synaptic vesicles and recycling endosomes cannot be differentially targeted for fusion by their respective v-SNAREs as proposed in the original presentation of the SNARE hypothesis.

After exocytosis, synaptic vesicle membranes recycle within the nerve terminal (Holtzman et al., 1971). Endocytosis is certainly required for synaptic vesicle recycling, but the exact pathway of recycling is unknown. The simplest possible mechanism would be to reverse the opening of a transient fusion pore after neurotransmitters are released (Alvarez de Toledo et al., 1993). However, at least in some cases, synaptic vesicles do fuse completely with the plasma membrane (Heuser and Reese, 1973; von Gersdorff and Matthews, 1994). Clathrin-coated vesicles probably have a major role in synaptic vesicle recycling since the predominant membrane proteins identified in coated vesicles from nerve terminals are synaptic vesicle membrane proteins (Maycox et al., 1992). After disassembly of their clathrin coats, primary endocytotic vesicles generally fuse with endosomes. Endosomes could function as a sorting station in the vesicle recycling pathway where synaptic vesicle proteins are segregated away from other proteins that either recycle back to the plasma membrane or return to the cell body by retrograde axonal transport. Alternatively, transport through an endosomal intermediate may be unnecessary if synaptic vesicle proteins are segregated before endocytosis. Synaptic vesicles might recycle by more than one pathway in nerve terminals. Routine recycling could occur by budding from the plasma membrane immediately after recycling while missorted proteins and proteins newly delivered from the cell body are sorted to synaptic vesicles within endosomes (Jahn and Sudhof, 1993).

PC12 pheochromocytoma cells have vesicles analogous to synaptic vesicles and have been used for studies of synaptic vesicle biogenesis because of their accessibility and ability to express transfected proteins. PC12 synaptic vesicles contain all of the general synaptic vesicle membrane proteins and are similar in their physical properties to the synaptic vesicles of rat brain (Wiedenmann et al., 1988; Clift-O'Grady et al., 1990; Cameron et al., 1991). Synaptic vesicle proteins recycle from the plasma membrane to PC12 synaptic vesicles by a process that excludes markers

of other endocytotic pathways such as the transferrin receptor (Clift-O'Grady et al., 1990). Since a large fraction of the synaptophysin in PC12 cells is present in endosomal organelles that also contain the transferrin receptor, it has been proposed that PC12 synaptic vesicles bud from endosomes (Cameron et al., 1991; Linstedt and Kelly, 1991). In support of this proposal, a kinetic lag has been observed before the fluid phase endocytosis marker HRP can be detected in synaptic vesicles (Bauerfeind et al., 1993). Thus, at least in PC12 cells, synaptic vesicle membrane proteins are probably internalized to endosomes before they are sorted to synaptic vesicles. This two-step recycling process suggested to us that synaptic vesicle membrane proteins may have two distinct targeting signals that act sequentially.

CHO cells are commonly used to study the behavior of synaptic vesicle proteins in cells that lack synaptic vesicles. It is generally assumed that membrane traffic in CHO cells is identical to that in PC12 cells except for the absence of the machinery for biogenesis of synaptic vesicles and secretory granules. In previous studies of CHO cells transfected with synaptic vesicle proteins it has been reported that synaptophysin is targeted to early endosomes, synaptotagmin remains on the cell surface associated with focal adhesions, and SV2 is targeted to intracellular organelles distinct from those containing synaptophysin (Johnston et al., 1989; Feany and Buckley, 1993; Feany et al., 1993). When the three proteins are cotransfected they still target to distinct organelles and do not associate with each other or induce the formation of synaptic vesicles (Feany et al., 1993). Since CHO cells do not have the machinery for synaptic vesicle biogenesis it is uncertain whether they express the "sortase" that binds to the SVTS of VAMP. Although CHO cells would not be expected to recognize a signal dedicated for synaptic vesicle targeting, they might still recognize the SVTS since it is conserved in cellubrevin.

In this paper we report the results of our attempt to identify a signal for VAMP-TAG endocytosis using the comprehensive collection of mutations created for the identification of the SVTS. Instead of identifying a distinct endocytosis signal, we discovered that mutations in the SVTS inhibit endocytosis in PC12 cells in addition to synaptic vesicle targeting. We also found that endocytosis of VAMP-TAG in CHO cells is rapid and partially dependent on the SVTS. These results suggest that the SVTS is a general signal for v-SNARE recycling.

## Materials and Methods

### Cell Culture, Transfection, cDNAs, Antibodies, and Iodination

Conditions for growth and transfection of PC12 cells, construction of the wild-type and mutant VAMP-TAG expression vectors, and the purification and iodination of the KT3 monoclonal antibody have been previously described (Grote et al., 1995). CHO cells were grown in DME H-21 media supplemented with 5% fetal calf serum and were transfected by electroporation using 250  $\mu$ F capacitors charged to 300 mV. Expression vectors for the wild-type and mutant polymeric immunoglobulin receptor (pIgR) variants and F<sub>AB</sub> fragments of a polyclonal antibody to the luminal domain of the pIgR were the generous gift of C. Okamoto and K. Mostov (Okamoto et al., 1992). The anti-pIgR F<sub>AB</sub>'s were iodinated to 20  $\mu$ Ci/mg using the ICl method (Breitfeld et al., 1989).

## Immunofluorescent Labeling

PC12/VAMP-TAG cells were plated on poly-D-lysine-coated glass coverslips or on Matrigel (Collaborative Research) coated chamber slides 2 d before staining. To observe total VAMP-TAG staining, the cells were washed in PBS and fixed with 3% paraformaldehyde in PBS. Fixed cells were permeabilized with 0.1% Saponin in PBS/1% BSA, incubated with the KT3 monoclonal antibody in permeabilization buffer, and detected with fluorescein-conjugated goat anti-mouse IgG (Cappel). For the immunofluorescent endocytosis assay, cells were incubated with 2  $\mu$ g/ml KT3 in DME H-21, 20 mM Hepes, pH 7.4, for 5 min at 37°C, then washed with 125 mM NaCl, 20 mM glycine, pH 2.4, at 4°C to remove surface staining. To visualize internalized KT3, the cells were fixed, permeabilized, and stained with fluorescein-conjugated secondary antibodies as above.

## Endocytosis Measurements with NHS-SS-biotin

Cell surface proteins were labeled with NHS-SS-biotin (Pierce), warmed to 37°C for the indicated times, and then biotin remaining on the cell surface was removed by washing in glutathione (Lisanti et al., 1990). Cells were lysed in IP buffer (1% NP-40 0.5% deoxycholate, 150 mM NaCl, 10 mM Tris-HCl, pH 7.8) and insoluble aggregates were removed in a 15 min, 16,000 g pellet. Biotinylated proteins from clarified lysate equivalent to 1/4 of a 3.5-cm dish were collected with 50  $\mu$ l streptavidin-agarose beads (Sigma), eluted in nonreducing sample buffer and resolved by SDS-PAGE. VAMP-TAG was detected on immunoblots probed with KT3 antibodies using a chemiluminescent detection system (Amersham). KT3 (8  $\mu$ g/ml) was present during the washing and incubation steps where indicated.

## Endocytosis Measurements with Iodinated Antibodies

Transfected cells were replated on poly-D-lysine-coated dishes 1 d before the assay. Cells were incubated with up to  $10^7$  cpm/ml [ $^{125}$ I]-antibody (<2  $\mu$ g/ml) in PBS/5% BSA for 45 min at 4°C. Unbound antibody was removed by extensive washing in PBS/BSA at 4°C. The cells were next incubated in PBS/BSA at 37°C for various times to allow endocytosis and then returned to 4°C. Antibody remaining at the cell surface was removed by two 15-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 with IP buffer and then scraping them from the dish. The fraction of [ $^{125}$ I]-KT3 internalized was calculated by dividing the acid wash resistant radioactive counts per minute (cpm) by the sum of acid wash resistant and accessible cpm. 10% of the [ $^{125}$ I]KT3 was resistant to the acid stripping procedure in both cells left at 4°C and in paraformaldehyde fixed cells. This background was subtracted when comparing endocytosis rates of the VAMP-TAG mutants. No correction was made for the dissociation of [ $^{125}$ I]-KT3 from cells that inevitably occurs during the course of the assay. The rate of dissociation of [ $^{125}$ I]-KT3 increases from 0.3% per minute at 4°C to 2% per minute at 37°C. Since antibody that dissociates from VAMP-TAG within an endosome remains cell associated until the endosome recycles to the cell surface, whereas antibody that dissociates from VAMP-TAG at the plasma membrane is released immediately into the media, the calculated fraction of internal [ $^{125}$ I]KT3 is artificially high at late time points.

## Cell Surface Fraction Measurements with NHS-SS-Biotin

Cells were labeled at 4°C with NHS-SS-Biotin and a clarified lysate was prepared in IP buffer. VAMP-TAG and VAMP-2 were immunoprecipitated with the 69.1 anti-VAMP-2 antibody and protein G agarose beads and eluted from the precipitated complex by boiling for 5 min in 10% SDS. An aliquot of total VAMP immunoprecipitate was set aside. The remainder of the eluted immunoprecipitate was diluted 60-fold in 2.5% Triton X-100, 125 mM NaCl and biotinylated proteins were collected on streptavidin-agarose beads. Proteins were eluted from the streptavidin-agarose beads by boiling in sample buffer with 750 mM  $\beta$ -mercaptoethanol and resolved by SDS-PAGE. Total and biotinylated VAMP-TAG and VAMP-2 were detected on immunoblots with the 69.1 monoclonal antibody.

## Cell Surface Fraction Measurements with [ $^{125}$ I]KT3

Cells were plated on poly-L-lysine-coated dishes, fixed in 3% paraformaldehyde in PBS, and incubated with [ $^{125}$ I]-KT3 in PBS/5% BSA in the pres-

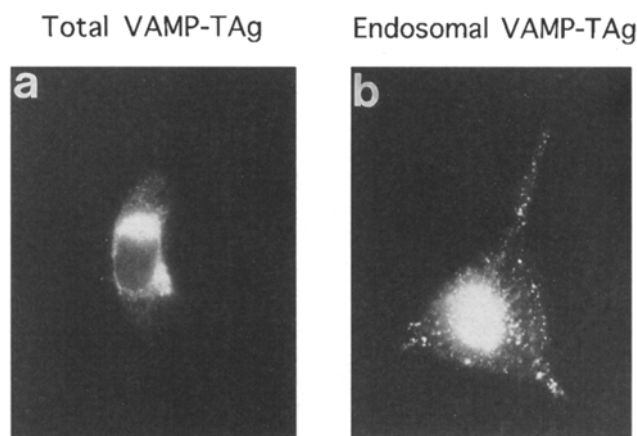
ence or absence of 0.05% saponin. After seven washes in PBS/BSA (with or without saponin), the cells were collected in IP buffer as above. The fraction on the cell surface was calculated by dividing the cpm bound to the surface of fixed cells by the cpm bound when internal KT3 binding sites were made accessible by permeabilization of membranes with saponin. Initial experiments demonstrated that the total cpm bound to cells increased proportionally to the saponin concentration until reaching a plateau at 0.04% saponin.

## Results

### Targeting and Transport of VAMP-TAG to Endosomes

VAMP-TAG was constructed by appending an 8-amino acid epitope from the SV-40 T antigen and a 14-amino acid spacer to the COOH terminus of rat VAMP-2 (Grote et al., 1995). Since only 2% of the total VAMP-TAG in transfected PC12 cells was recovered in synaptic vesicles, immunofluorescent microscopy was used to identify where the remainder of the transfected VAMP-TAG is targeted. Stably transfected PC12/VAMP-TAG cells (Grote et al., 1995) were stained with the KT3 monoclonal antibody, which binds to the epitope tag (MacArthur and Walter, 1984). In saponin-permeabilized cells VAMP-TAG was observed in punctate organelles concentrated in the perinuclear region of the cell and at the cell surface (Fig. 1). Surface staining was more apparent in nonpermeabilized cells (data not shown). No staining was observed in nontransfected cells.

The distribution of VAMP-TAG in PC12 cells is similar to the staining pattern of synaptophysin and the transferrin receptor, two proteins that colocalize in early endosomes (Cameron et al., 1991). To determine if VAMP-TAG is also found in an endosome, VAMP-TAG transfected PC12 cells were incubated with KT3 monoclonal antibody for 5 min at 37°C. Antibody remaining bound to the cell surface was removed by a low pH wash at 4°C and the cells were fixed, permeabilized, and then stained with fluorescent secondary antibodies. Internalized VAMP-TAG was observed in punctate organelles concentrated in the perinuclear region similar to those stained in permeabilized



**Figure 1.** Immunofluorescent labeling of steady-state and internalized VAMP-TAG in PC12 cells. (a) Total VAMP-TAG was stained by indirect immunofluorescence using the KT3 mAb. (b) To detect endosomal VAMP-TAG, cells were incubated with KT3 mAb for 5 min at 37°C and washed in acidic buffer at 4°C before fixation.

cells (Fig. 1). Again, no staining was observed in nontransfected cells. The similarity in the staining patterns of VAMP-TAg at steady-state and after internalization suggests that a large fraction of VAMP-TAg is targeted to endosomes in transfected PC12 cells.

KT3 is a bivalent antibody and might potentially cross-link VAMP-TAg and alter its subcellular distribution. In lymphocytes, cross-linking induces capping and internalization (Schreiner and Unanue, 1976). An attempt was made to observe VAMP-TAg endocytosis using monovalent  $F_{AB}$  fragments of the KT3 monoclonal antibody to exclude the possibility that the VAMP-TAg internalization observed above was artificially induced by cross-linking. Unfortunately, the affinity of the KT3  $F_{AB}$ 's was insufficient to observe internalization. An alternate approach was therefore adopted using the membrane impermeable surface labeling reagent NHS-SS-biotin to determine the effect of KT3 on VAMP-TAg endocytosis. Biotinylation of VAMP-TAg is unlikely to affect KT3 binding since the KT3 epitope does not contain any free amino groups with the potential to react with NHS-SS-biotin. Proteins at the cell surface of VAMP-TAg transfected PC12 cells were biotinylated at 4°C, then warmed to 37°C in the presence or absence of bivalent KT3 at four times the concentration used to observe endocytosis. At various time points the cells were returned to 4°C and biotin was stripped from proteins remaining at the cell surface by reduction of the disulfide bond with glutathione. Internalized VAMP-TAg was collected on streptavidin-agarose beads and detected on immunoblots with KT3 (Fig. 2). The results demonstrate that VAMP-TAg internalization was not induced by KT3 antibodies. Thus, bivalent KT3 can be used to measure the basal rate of VAMP-TAg internalization.

### Endocytosis of VAMP-TAg Is Rapid and Saturable in PC12 Cells

A quantitative assay using iodinated KT3 was developed to measure the rate of VAMP-TAg endocytosis. For this assay, the pool of VAMP-TAg on the plasma membrane was labeled at 4°C with [ $^{125}$ I]-KT3. Binding of [ $^{125}$ I]-KT3 to VAMP-TAg at the cell surface is specific since 15 times more [ $^{125}$ I]-KT3 bound to PC12/VAMP-TAg cells than to nontransfected PC12 cells. After warming to 37°C for various times [ $^{125}$ I]-KT3 remaining at the cell surface was removed by a low pH wash and the fraction of antibody resistant to the acid wash due to endocytosis was calculated. Internalization of [ $^{125}$ I]-KT3 in PC12/VAMP-TAg cells

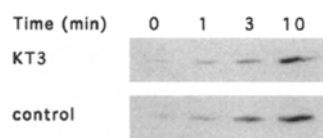


Figure 2. VAMP-TAg endocytosis occurs independently of KT3. Proteins on the plasma membrane of PC12/VAMP-TAg cells were labeled at 4°C with the membrane impermeable reagent NHS-SS-biotin. The cells were then incubated with or without KT3 antibodies at four times the standard concentration. After incubation at 37°C for the indicated times (in minutes), biotin was stripped from proteins remaining at the cell surface with glutathione. Biotinylated proteins protected from reduction by endocytosis were collected on streptavidin-agarose, and internalized VAMP-TAg was detected by immunoblotting with KT3 antibodies.

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was rapid, proceeding at an initial rate of 19% per minute (Fig. 3).

Internalization of [ $^{125}$ I]-KT3 was also measured in PC12 cells transiently transfected with VAMP-TAg by electroporation. Compared with the clonal PC12/VAMP-TAg cell line, the rate of VAMP-TAg endocytosis was relatively slow in transiently transfected PC12 cells. One possible explanation for this observation is that the pathway for VAMP-TAg endocytosis is saturated at high expression levels. To determine if the expression level of VAMP-TAg in transiently transfected cells is high enough to potentially saturate the endocytosis pathway, the expression level of VAMP-TAg was compared with the native expression level of VAMP-2. The mean expression level of VAMP-TAg in PC12 cells transfected with 75  $\mu$ g of the pRC/CMV/VAMP-TAg expression vector is approximately twofold greater than that of VAMP-2, and similar to the expression level of VAMP-TAg in the PC12/VAMP-TAg cell line. However, only 20% of the PC12 cells that survive electroporation express enough VAMP-TAg to be detected by immunofluorescent staining (data not shown). Thus, the mean expression level in the subset of cells expressing VAMP-TAg after transient transfection is elevated to  $\sim$ 10 times the expression level of VAMP-2.

To measure the effect of expression level on the rate of VAMP-TAg endocytosis, internalization of [ $^{125}$ I]-KT3 was assayed in PC12 cells transiently transfected with varied amounts of the pRC/CMV/VAMP-TAg expression vector. Increasing the amount of VAMP-TAg cDNA resulted in increased binding of [ $^{125}$ I]-KT3 to the cell surface of transfected cells (Fig. 4 a). The fraction of [ $^{125}$ I]-KT3 internalized to an acid resistant fraction during 3 min at 37°C was inversely proportional to the VAMP-TAg expression level (Fig. 4 b). This result indicates that the endocytosis pathway is saturable and implies that a limiting component is required for rapid endocytosis. At the highest total expression level, the fraction of [ $^{125}$ I]-KT3 internalization in 3 min did not fall below 0.2. This suggests that there may also be a slow, nonsaturable VAMP-TAg endocytosis pathway. However, since populations of transiently trans-

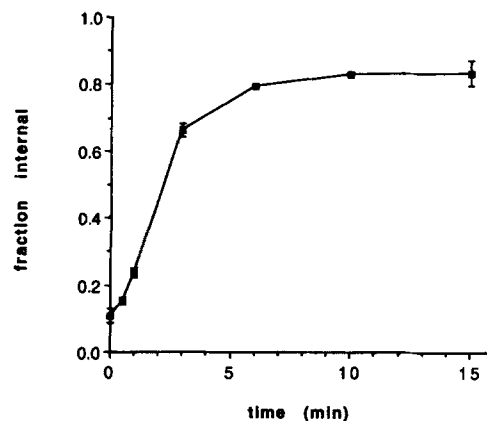
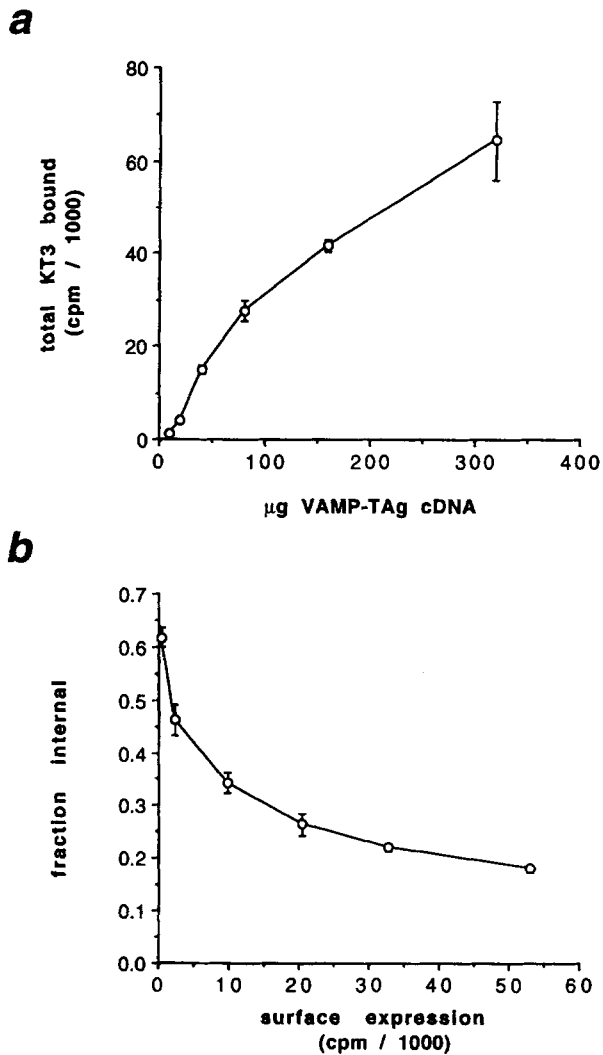


Figure 3. Quantitative measurement of VAMP-TAg endocytosis in PC12 cells. Internalization measurements were made by determining the fraction of radioiodinated antibody that was internalized to an endosomal compartment resistant to an acid wash. Error bars represent the standard deviation,  $n = 3$ . Deviations of less than 0.015 are not displayed.



**Figure 4.** Endocytosis of VAMP-TAg is saturable. (a) Titration of VAMP-TAg expression. PC12 cells were transiently transfected by electroporation using various amounts of the pRC/CMV/VAMP-TAg cDNA expression vector. Surface expression was measured by [<sup>125</sup>I]-KT3 binding. (b) VAMP-TAg overexpression inhibits endocytosis. Endocytosis of VAMP-TAg was measured after 3 min at 37°C. Measurements were made as in Fig. 3 except that background counts from [<sup>125</sup>I]-KT3 binding to mock-transfected cells were subtracted.

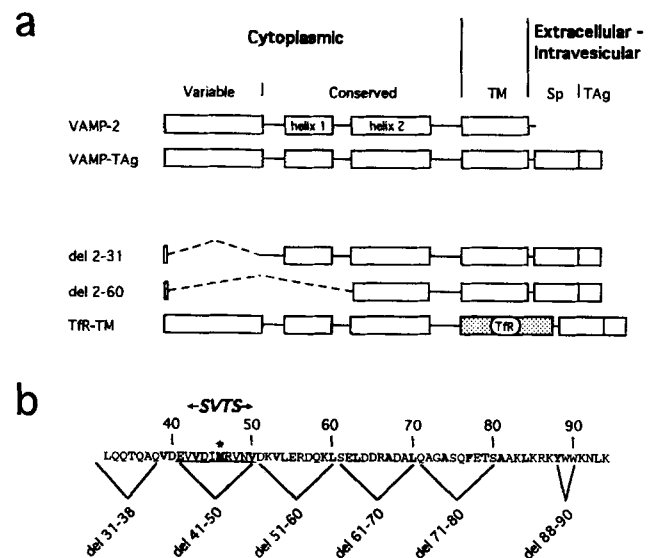
fectected cells do not have a uniform expression level, an alternate explanation for the residual internalization at high expression levels is that even if the amount of cDNA transfected exceeds 100 µg, a certain fraction of the VAMP-TAg molecules are still expressed in cells where the expression level is below that which saturates endocytosis.

#### Involvement of the Synaptic Vesicle Targeting Signal in Endocytosis of VAMP-TAg in PC12 Cells

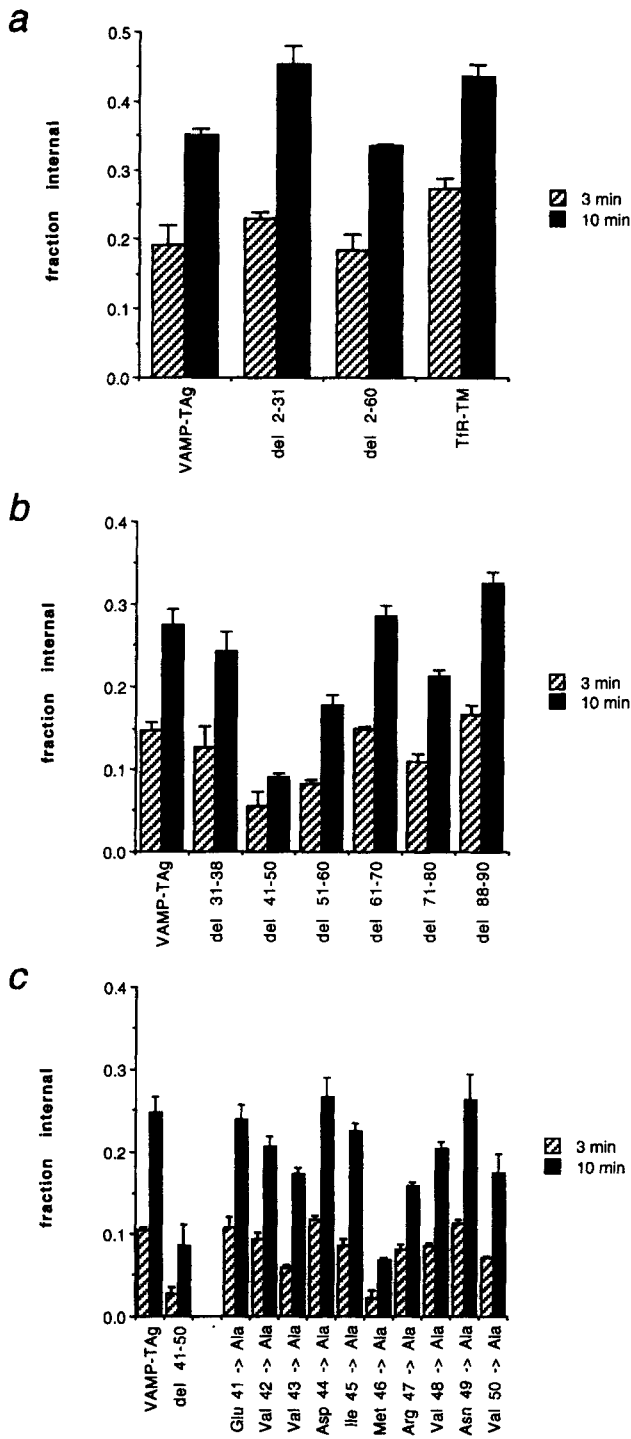
VAMP-TAg does not contain any sequence motifs with obvious homology to previously identified signals for coated pit-mediated endocytosis. Therefore, endocytosis rates were measured for members of a comprehensive collection of VAMP-TAg mutants to identify sequence mo-

tifs required for endocytosis (Fig. 5). Endocytosis rates were first compared in transiently transfected PC12 cells. An advantage of using transiently transfected cells is that they have a uniform growth rate and do not display clonal variability in morphological properties. For the wild-type VAMP-TAg construction, 40 µg of cDNA was used for the transfection to give adequate protein expression without saturating the endocytosis pathway. The amount of each mutant VAMP-TAg cDNA used was adjusted to provide approximately equal levels of cell surface expression. Similar concentrations of cDNA were used for expression of all of the mutants with the exception of del 2-60.

The first set of mutants examined for internalization competence includes two large NH<sub>2</sub>-terminal deletions (del 2-31 and del 2-60) and a chimera in which the transmembrane domain of VAMP-TAg is replaced by the transmembrane domain of the transferrin receptor (TfR-TM). None of these mutants inhibited endocytosis (Fig. 6 a). Since the endocytosis rates for the del 2-31 and TfR-TM mutants are actually faster than that of VAMP-TAg, the NH<sub>2</sub>-terminal and transmembrane domains may inhibit endocytosis. The rapid endocytosis rate of the del 2-60 mu-



**Figure 5.** Structure of VAMP-TAg and the mutants. (a) Schematic representation of VAMP-TAg and the domain mapping mutations. The cytoplasmic domain of VAMP-2 consists of an NH<sub>2</sub>-terminal variable domain and a conserved domain containing two predicted amphipathic α-helices. To create VAMP-TAg, an 8-amino acid epitope from the SV-40 T antigen was appended, via a 14-amino acid spacer, to the COOH terminus of the transmembrane anchor. The epitope tag is accessible to antibody in the media when VAMP-TAg is on the plasma membrane. Two large NH<sub>2</sub>-terminal deletions and a chimera containing the transmembrane domain of the transferrin receptor were constructed to identify targeting domains. (b) Mutations within the conserved domain of VAMP-TAg. The sequence of the conserved domain is shown with amino acids on the hydrophobic face of the two predicted amphipathic α-helices indicated in bold type. The synaptic vesicle targeting signal (SVTS) is contained within helix 1 and is centered on methionine-46 (\*). Amino acids removed in each internal deletion are indicated. The underlined amino acids (41-50) were each mutated, individually, to alanine.

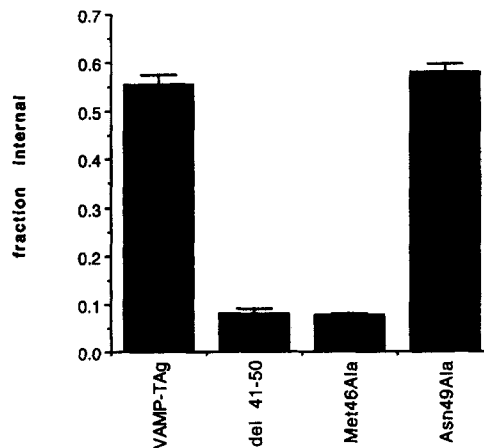


**Figure 6.** Internalization rates for the VAMP-TAg mutants in PC12 cells. (a) Domain mapping mutations. (b) Internal deletions within the conserved domain. (c) Alanine scanning mutants within the synaptic vesicle targeting signal. The internalization of [<sup>125</sup>I]-KT3 was measured as in Fig. 4 except that a time = 0 background of between 0.06 and 0.12 was subtracted for each mutant.

tant suggests that the synaptic vesicle targeting signal (SVTS) is not required for endocytosis, but this result must be cautiously interpreted since it was necessary to use 300 μg of del 2-60 cDNA to have adequate surface expression.

Endocytosis was next measured for a series of VAMP-TAg mutants with internal deletions within the conserved domain. Included in this group is del 88-90, a mutant that lacks the only tyrosine in the cytoplasmic domain. Among the internal deletions, the strongest inhibition of endocytosis was observed for del 41-50, a deletion of the central core of the SVTS (Fig. 6 b). Endocytosis was also partially inhibited by del 51-60, a deletion that lacks the COOH terminus of the predicted amphipathic helix containing the SVTS. Internalization of a series of VAMP-TAg mutants in which amino acids 41-50 were each substituted with alanine was measured to further define the relationship between the SVTS and endocytosis. The Met<sub>46</sub>Ala mutant, the most potent inhibitor of synaptic vesicle targeting, inhibited endocytosis by 80% (Fig. 6 c). Mutation of Val<sub>43</sub> or Val<sub>50</sub>, amino acids adjacent to Met<sub>46</sub> on the hydrophobic face of the predicted amphipathic helix comprising the SVTS, also inhibited endocytosis. In contrast to the mutations that blocked synaptic vesicle targeting, mutations such as del 61-70 and Asn<sub>49</sub>Ala that enhanced targeting to synaptic vesicles did not affect the endocytosis rate. This result provides indirect support that [<sup>125</sup>I]-KT3 is internalized primarily to endosomes rather than to synaptic vesicles.

Internalization of selected VAMP-TAg mutants was also compared in clonal PC12-derived cell lines. In contrast to transiently transfected cells, expression in cell lines is not limited to a minority of cells with relatively high expression. The clonal cell lines selected for analysis expressed the del 41-50, Met<sub>46</sub>Ala and Asn<sub>49</sub>Ala mutants at levels similar to the expression level of VAMP-TAg in the PC12/VAMP-TAg cell line. The relative endocytosis rates of the mutants in the clonal cell lines paralleled the rates observed after transient transfection (Fig. 7). Endocytosis was slow for the del 41-50 and Met<sub>46</sub>Ala mutants but unchanged for Asn<sub>49</sub>Ala. Thus, the reduction in endocytosis rates observed with mutations in the SVTS that inhibit synaptic vesicle targeting is not an artifact resulting from overexpression of the mutant proteins by transient transfection.



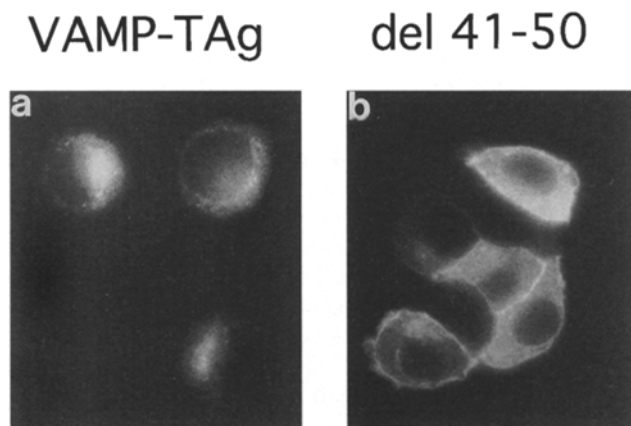
**Figure 7.** Internalization of VAMP-TAg mutants in clonal PC12-derived cell lines. Cell lines were selected that expressed VAMP-TAg proteins with mutations that affect synaptic vesicle targeting at expression levels similar to that of VAMP-2. Internalization of [<sup>125</sup>I]-KT3 after 3 min at 37°C was measured as in Fig. 3.

### Defects in Endocytosis Result in Enhanced Surface Accessibility

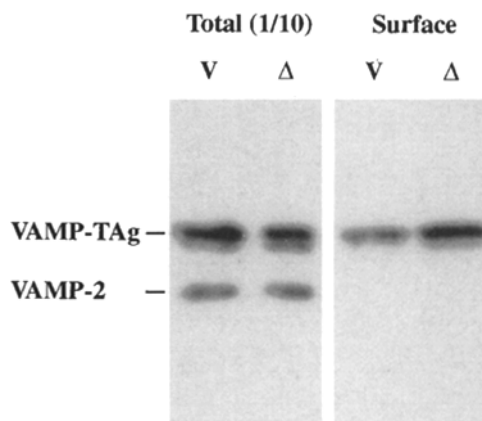
If reductions in the rate of VAMP-TAg endocytosis result from defects specific to the endocytotic stage of the recycling pathway, then VAMP-TAg should accumulate on the plasma membrane when its endocytosis is inhibited. This effect was first observed during screening to identify a clonal cell line that expresses the del 41-50 mutation by immunofluorescent staining. Compared to PC12/VAMP-TAg cells, PC12-derived cell lines transfected with the del 41-50 mutant have enhanced surface staining with the KT3 antibody (Fig. 8).

The accumulation of the del 41-50 mutant at the plasma membrane was confirmed by a cell surface biotinylation assay. Proteins on the cell surface of PC12/VAMP-TAg and PC12/del 41-50 cells were biotinylated at 4°C with NHS-SS-Biotin. The total population of VAMPs was immunoprecipitated with a VAMP-2-specific antibody, eluted with SDS, and the fraction of VAMP-TAg from the plasma membrane was reprecipitated with streptavidin-agarose beads. Aliquots of the total immunoprecipitate and streptavidin-agarose precipitate were resolved on a reducing polyacrylamide gel and an immunoblot was probed with the anti-VAMP-2 antibody (Fig. 9). The immunoprecipitated VAMP-2 serves an internal control. Since VAMP-2 lacks reactive amines accessible from outside the cell it cannot be biotinylated. Thus, the absence of VAMP-2 from the streptavidin-agarose precipitates demonstrates the specificity of this method. The results show that the total expression level of VAMP-TAg (V) and the del 41-50 mutant ( $\Delta$ ) are similar, but that there is more del 41-50 on the cell surface. Thus, the surface staining observed for del 41-50 in individual cells by immunofluorescence reflects enhanced accumulation on the plasma membrane in the entire population of transfected cells.

To quantify the fraction of VAMP-TAg on the cell surface, the accessibility of the T antigen epitope to [<sup>125</sup>I]-KT3 in paraformaldehyde-fixed cells was compared in the absence or presence of saponin, a detergent that permeabilizes cell membranes. 3% of the total [<sup>125</sup>I]-KT3 binding sites in PC12/VAMP-TAg cells are accessible in the ab-



**Figure 8.** Enhanced surface staining of PC12 cells transfected with the del 41-50 mutant. Immunofluorescent staining with the KT3 monoclonal antibody of saponin-permeabilized PC12 clones transfected with (a) VAMP-TAg or (b) del 41-50.



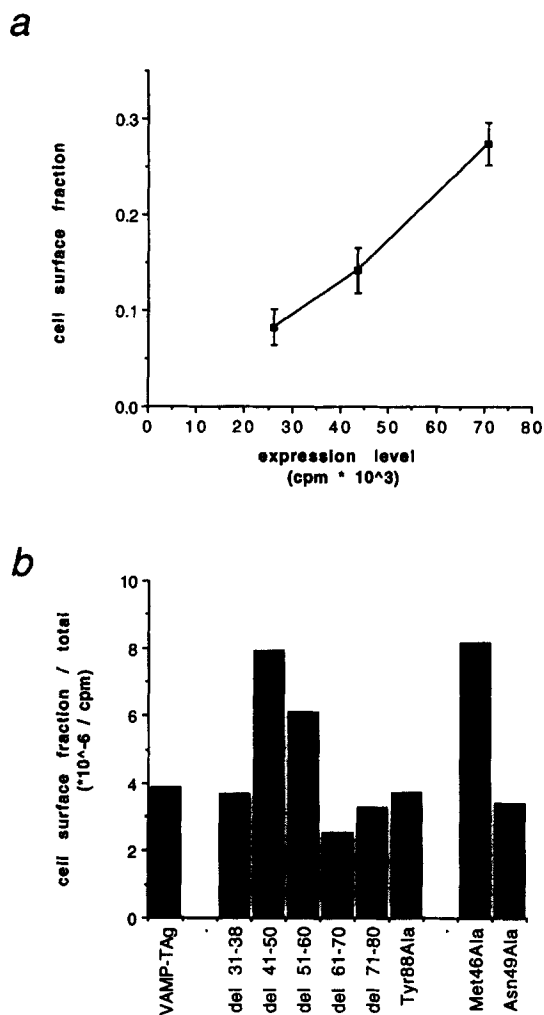
**Figure 9.** Accumulation of the del 41-50 mutant on the surface of PC12 cells. Proteins on the cell surface of clonal PC12-derived cell lines expressing VAMP-TAg (V) or the del 41-50 mutant ( $\Delta$ ) were labeled at 4°C with NHS-SS-Biotin. VAMPs were collected by immunoprecipitation with an anti-VAMP-2-specific monoclonal antibody and eluted from the precipitate with 10% SDS. An aliquot of the total VAMP immunoprecipitate was set aside and the remainder of the sample was reprecipitated with streptavidin-agarose beads and eluted in reducing sample buffer. An immunoblot of the total VAMP precipitates and the biotinylated surface fractions was probed with the anti-VAMP-2 antibody.

sence of saponin. The quantitative results with this [<sup>125</sup>I]-KT3 binding assay are consistent with the qualitative observations made with the cell surface biotinylation assay (data not shown). The fraction of [<sup>125</sup>I]-KT3 binding sites accessible in the absence of saponin increased when endocytosis was inhibited by overexpression (Fig. 10 a). Similarly, enhanced accumulation at the cell surface of transiently transfected PC12 cells was observed for those mutations in the SVTS, such as del 41-50 and Met<sub>46</sub>Ala, that inhibit endocytosis (Fig. 10 b). Thus, both overexpression of VAMP-TAg and mutation of the SVTS predominantly inhibit endocytosis rather than recycling to the plasma membrane.

### VAMP-TAg Endocytosis in CHO Cells

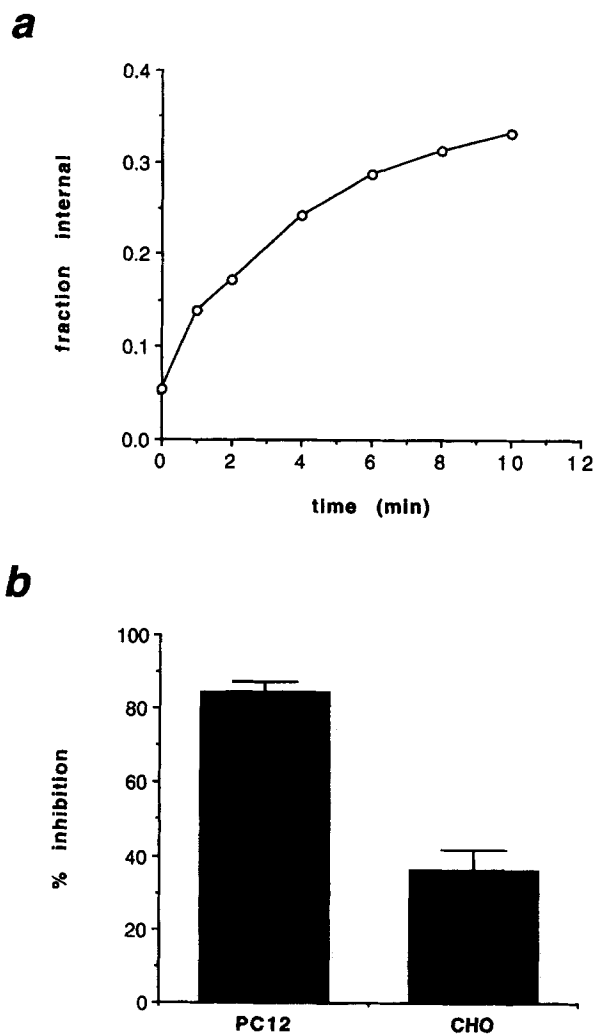
To determine if neuroendocrine-specific components such as other synaptic vesicle proteins or specialized components of clathrin-coated pits are required for VAMP-TAg endocytosis, internalization was measured in transiently transfected CHO cells (Fig. 11 a). The rapid initial rate of VAMP-TAg internalization in CHO cells implies that VAMP-TAg contains a cell type-independent endocytosis signal. The role of the SVTS in VAMP-TAg endocytosis in CHO cells was tested by comparing internalization of the wild-type and Met<sub>46</sub>Ala mutant VAMP-TAg proteins. The Met<sub>46</sub>Ala mutation inhibited endocytosis by 35% (Figure 11 b). The inhibitory effect of the mutation demonstrates that CHO cells express components that can interact with the SVTS. However, the 35% inhibition in CHO cells is small compared to the 85% inhibition of endocytosis observed for the Met<sub>46</sub>Ala mutation in PC12 cells.

The cell type-dependent differences in the extent of in-



**Figure 10.** Accessibility of VAMP-TAG to [<sup>125</sup>I]-KT3 surface binding. (a) Overexpressed VAMP-TAG accumulates at the cell surface. The fraction of VAMP-TAG on the cell surface was measured for PC12 cells transiently transfected with various concentrations of cDNA. Cells were fixed with paraformaldehyde, incubated in [<sup>125</sup>I]-KT3 labeling media with or without 0.05% saponin, and then washed. The fraction of [<sup>125</sup>I]-KT3 binding sites on the cell surface was calculated by dividing the cpm bound to cells in the absence of saponin by the cpm bound with saponin. (b) VAMP-TAG mutants defective for endocytosis accumulate at the cell surface. PC12 cells were transiently transfected with 40 μg cDNA coding for selected VAMP-TAG mutants. To normalize for subtle variations in expression level, the fraction of surface accessible [<sup>125</sup>I]-KT3 binding sites was divided by the total [<sup>125</sup>I]-KT3 cpm bound. Accumulation of the del 41-50 and Met<sub>46</sub>Ala mutants on the cell surface was apparent with or without this correction. Error bars represent the standard error of the mean, *n* = 3.

inhibition by the Met<sub>46</sub>Ala mutation might result from differences between the two cell types in the basic machinery for endocytosis. To examine this possibility, a control experiment was performed using another rapidly internalized membrane protein, the polymeric immunoglobulin receptor (pIgR). The pIgR is normally expressed in mucosal epithelia and has two strong tyrosine-dependent endocytosis signals in its cytoplasmic domain (Okamoto et al., 1992). A Tyr<sub>668</sub>Cys, Tyr<sub>734</sub>Ser double mutation destroys



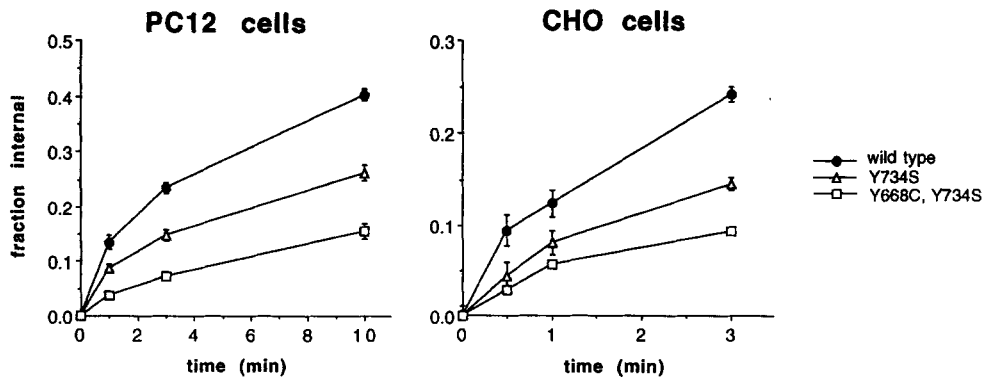
**Figure 11.** The SVTS promotes VAMP-TAG endocytosis in CHO cells. (a) Endocytosis of VAMP-TAG in transiently transfected CHO cells was measured as in Fig. 3. (b) Inhibition of VAMP-TAG endocytosis by the Met<sub>46</sub>Ala mutation in CHO and PC12 cells. The fraction of [<sup>125</sup>I]-KT3 internalized during 3 min at 37°C was measured for cells transfected with VAMP-TAG and the Met<sub>46</sub>Ala mutant and the percentage of endocytosis inhibited by the mutation was calculated. Six independent experiments were performed with CHO cells and three independent experiments were performed with PC12 cells. Error bars represent the standard error of the mean.

these two endocytosis signals. pIgR internalization was inhibited by 57% in both PC12 and CHO cells by this mutation (Fig. 12). Mutation of only the Tyr<sub>734</sub> signal had an intermediate effect of identical magnitude in both cell types. These results imply that the basic machinery for internalization and recognition of standard endocytosis signals is identical in PC12 and CHO cells and cannot account for the differential effect of the Met<sub>46</sub>Ala mutation on VAMP-TAG endocytosis in the two cell types.

## Discussion

Synaptic vesicle proteins present on the presynaptic plasma membrane after exocytosis are rapidly internalized





**Figure 12.** Endocytosis of the pIgR is inhibited to an equivalent extent in PC12 and CHO cells by mutations in tyrosine-dependent endocytosis signals. PC12 and CHO cells were transfected with expression vectors for the wild-type and mutant forms of the pIgR. Endocytosis was measured as in Fig. 6 with radioiodinated F<sub>AB</sub> fragments of a polyclonal pIgR antibody.

via coated pits to regenerate synaptic vesicles (Miller and Heuser, 1984; Matteoli et al., 1992; Maycox et al., 1992). Endocytosis of VAMP-2 cannot be measured, however, because it cannot be labeled from outside the cell by membrane impermeable reagents since it does not have an extracellular domain. To overcome this difficulty, a modified form of VAMP-2 was constructed with a COOH-terminal T antigen epitope tag and a spacer containing three lysines. The engineered protein, VAMP-TAg, was labeled at the surface of transfected cells and tracked with remarkably consistent results using two independent techniques, binding of the monoclonal antibody KT3 and covalent coupling of biotin. The use of radioiodinated KT3 allows for a quantitative assay for VAMP-TAg endocytosis because over 90% of the antibody can be stripped from the cell surface by an acidic wash. Endocytosis of VAMP-TAg in PC12 cells is rapid and insensitive to potential cross-linking by bivalent KT3.

The rapid rate of VAMP-TAg endocytosis in PC12/VAMP-TAg cells is consistent with the expected pathway of internalization via clathrin coated pits. Further support for the involvement of coated pits is the observation that inhibition of clathrin assembly by incubation of VAMP-TAg transfected CHO cells in hypertonic media (Hansen et al., 1993) inhibits [<sup>125</sup>I]-KT3 internalization by greater than 90% (unpublished observation). There is, however, no direct morphological evidence for clustering of VAMP-TAg in coated pits.

VAMP-TAg endocytosis is retarded if the transfected protein is expressed at levels substantially higher than the native expression level of VAMP-2. Inhibiting endocytosis of VAMP-TAg by overexpression causes an accumulation of the protein on the plasma membrane indicating that transport of VAMP-TAg to the plasma membrane is not similarly inhibited. This result implies that a component with limited availability is required for rapid VAMP-TAg endocytosis. The limiting factor might be a protein with specificity for VAMP-TAg or a general component of the endocytosis machinery.

The saturation hypothesis can explain some puzzling observations about other synaptic vesicle proteins. For example, the fraction of synaptophysin that is internalized in PC12 cells is less than that of the transferrin and mannose-6-phosphate receptors (Green and Kelly, 1992) and also less than the extent of synaptophysin endocytosis in CHO cells (Linstedt and Kelly, 1991). These observations might be related to the fact that synaptophysin is naturally ex-

pressed at high levels compared with other synaptic vesicle proteins in PC12 cells (Clift-O'Grady et al., 1990). Saturation may also explain why synaptotagmin, a protein known to have a high affinity binding site for the AP2 adaptor protein complex of clathrin coated pits (Zhang et al., 1994), is predominantly found associated with actin-rich filopodia on the surface of transfected CHO cells (Feany and Buckley, 1993). A similar staining pattern was observed for VAMP-TAg in a CHO cell line with high expression (unpublished observation).

#### *Intracellular Distribution of VAMP-TAg*

We have previously reported that VAMP-TAg is targeted to synaptic vesicles in transfected PC12 cells. However, only a small fraction of the total protein is recovered in the synaptic vesicle peak (Grote et al., 1995). Immunofluorescent microscopy of transfected PC12 cells shows that VAMP-TAg is targeted to punctate organelles concentrated around the nucleus. KT3 monoclonal antibody bound to VAMP-TAg at the cell surface and internalized for 5 min is sorted to organelles with an intracellular distribution similar to that of VAMP-TAg at steady-state suggesting that the protein is primarily targeted to early endosomes. Although similar staining patterns have also been observed for the transferrin receptor (an early endosome marker) and for synaptophysin (a synaptic vesicle protein that is also found in early endosomes) no definitive conclusions can be drawn from this data because of the low resolution of immunofluorescent microscopy and the rounded shape of PC12 cells.

Other organelles that might contain VAMP-TAg include vesicles of the constitutive and regulated secretory pathways. VAMP has recently been shown to be a substrate for a novel SRP-independent mechanism for inserting COOH-terminal anchored proteins into the endoplasmic reticulum (Kutay et al., 1995). In agreement with this report, we have found that an O-linked glycan is added to a site in the spacer domain of VAMP-TAg with kinetics consistent with modification in the endoplasmic reticulum and Golgi apparatus as VAMP-TAg is transported through the secretory pathway (unpublished observation). However, only a small fraction of the total VAMP-TAg at steady state is likely to be in early compartments of the secretory pathway since the fraction of VAMP-TAg that is glycosylated reaches a plateau within 1 h after synthesis whereas the half-life of the protein is greater than 2 d. An-

other pool of VAMP-TAg may be stored in secretory granules since a fraction of the VAMP-2 endogenously expressed by PC12 cells is targeted to secretory granules (Chilcote et al., 1995; Papini et al., 1995). None of the VAMP-TAg mutations blocked transport through the secretory pathway to the cell surface.

### **Role of the SVTS in Endocytosis**

The central finding of this study is that the only mutations in VAMP-TAg that inhibit endocytosis are located within the predicted amphipathic  $\alpha$ -helix centered on methionine-46 that we have previously identified as the synaptic vesicle targeting signal (SVTS). Endocytosis of VAMP-TAg was inhibited by deleting segments of the helix or by substituting alanine for amino acids on its hydrophobic face. The SVTS of VAMP-TAg is unlike previously identified endocytosis signals. It is distinct from the tyrosine-based signals of the transferrin and LDL receptors because it does not contain aromatic amino acids and is predicted to form an  $\alpha$ -helix, not a  $\beta$ -turn. The difference between the SVTS and the dileucine signals found in CD3 and the MHC class II invariant chain is more subtle. The sequence of the SVTS fits the consensus for di-leucine signals because it has an acidic amino acid, glutamate-41, four amino acids NH<sub>2</sub>-terminal of a di-hydrophobic pair (Pond et al., 1995). However, alanine scanning mutagenesis of di-leucine signals does not result in a helical pattern of inhibition. Furthermore, the Glu<sub>41</sub>Ala and Ile<sub>45</sub>Ala mutations of VAMP-TAg did not inhibit endocytosis.

The unique features of the SVTS as an endocytosis signal allow for the possibility that the SVTS may promote endocytosis by a novel mechanism rather than binding to adaptors in coated pits. We previously suggested that the SVTS is required for binding to another synaptic vesicle protein that escorts VAMP-TAg to synaptic vesicles. Similarly, we now propose that VAMP binds, via the SVTS, to an escort protein that carries it from the cell surface to endosomes. Colocalization of the signals for endocytosis and synaptic vesicle targeting suggests that a single escort protein remains bound to VAMP-TAg throughout the synaptic vesicle recycling process. One implication of this model is that the principal interaction required for targeting VAMP-TAg to synaptic vesicles occurs at the cell surface. Alternatively, there may be a family of v-SNARE escort proteins that bind to the SVTS. In PC12 cells, one escort protein would be responsible for endocytosis and another for synaptic vesicle targeting. The synaptic vesicle escort could bind to VAMP-TAg either in endosomes or at the plasma membrane. A third escort protein could be responsible for endocytosis of both VAMP-TAg and cellubrevin in CHO cells. This multiple escort protein model can readily account for the differences between the effects on endocytosis and synaptic vesicle targeting of some mutations in the SVTS. For example, the Asn<sub>49</sub>Ala mutation enhances synaptic vesicle targeting, but does not affect endocytosis. Also, the Val<sub>50</sub>Ala mutation completely blocks synaptic vesicle targeting but only partially inhibits endocytosis. If there are multiple escort proteins, binding to the synaptic vesicle escort is likely to be more sensitive to inhibition by asparagine-49 or mutations on the hydrophobic face of the helix. Similarly, the escort protein ex-

pressed in CHO cells could be less sensitive to the Met<sub>46</sub>Ala mutation than its counterpart in PC12 cells.

The differential effects of some mutations on endocytosis and synaptic vesicle targeting can also be explained by elaborating the single escort protein model. The main feature of the new model is that VAMP-TAg will recycle to the plasma membrane before it is sorted to synaptic vesicles if it dissociates from its escort in endosomes. Thus, the Asn<sub>49</sub>Ala mutant is sorted more efficiently to synaptic vesicles because it has a higher affinity for the escort protein, but the Val<sub>50</sub>Ala mutant is internalized but not targeted to synaptic vesicles because its affinity for the escort protein is low. An additional postulate of this model is that the affinity of wild-type VAMP-TAg for the escort protein is already sufficient for the maximum possible rate of endocytosis. Therefore, the enhanced binding affinity of the Asn<sub>49</sub>Ala mutant does not increase its rate of endocytosis.

The incomplete inhibition of endocytosis in CHO cells by the Met<sub>46</sub>Ala mutation indicates that there are cell type-dependent differences in endocytosis of VAMP-TAg. Differences in the basic machinery for endocytosis cannot be responsible for this observation because mutation of the tyrosine-dependent endocytosis signals of the pIgR has an equivalent inhibitory effect on the internalization rate in PC12 and CHO cells. Cell type-specific differences in the effect of mutations on endocytosis have also been observed for a tyrosine-dependent signal in the LDL receptor (Davis et al., 1987; Matter et al., 1994) and a dileucine-based signal in the invariant chain (Odorizzi et al., 1994; Pond et al., 1995), but the cause of these differences is unknown. One explanation for the cell type-dependent differences in endocytosis of the Met<sub>46</sub>Ala VAMP-TAg mutant is that CHO and PC12 cells express different v-SNARE escort proteins as suggested earlier. Alternatively, if both cell types use the same v-SNARE escort protein for endocytosis, VAMP-TAg might have a secondary endocytosis signal active only in CHO cells that is responsible for endocytosis of the Met<sub>46</sub>Ala mutant. This secondary endocytosis signal could be inactive in PC12 cells either because PC12 cells do not express the appropriate endocytosis machinery or because its activity is inhibited. The enhanced internalization rate observed for the del 2-31 mutant suggests that the NH<sub>2</sub>-terminal variable domain could be responsible for inhibiting endocytosis. Absence of an NH<sub>2</sub>-terminal inhibitory domain can also explain the rapid internalization without an SVTS by the del 2-60 mutant in PC12 cells.

The overlap that we have discovered between the endocytosis and synaptic vesicle targeting signals in the cytoplasmic domain of VAMP-TAg is analogous to the overlap that has previously been reported between endocytosis signals and signals for targeting either to the basolateral surface of polarized epithelial cells or to late endosomes. The relationship between overlapping signals for endocytosis and basolateral targeting in the Fc and LDL receptors has been extensively studied (Matter et al., 1994). In the case of the Fc receptor the two dileucine signals are apparently identical. In contrast, the overlapping signals in the LDL receptor both require a critical tyrosine but are otherwise completely distinct. Many of the mutations in the cytoplasmic domain of VAMP-TAg have different effects on endocytosis and synaptic vesicle targeting, but

thus far the differences observed have been quantitative rather than qualitative. Thus, it is not certain whether a single escort protein binding to the SVTS can promote both endocytosis and synaptic vesicle targeting or whether multiple escort proteins are necessary. Future experiments will be directed towards the identification of proteins that interact with VAMP-TAG via the SVTS.

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