Synaptophysin I controls the targeting of VAMP2/synaptobrevin II to synaptic vesicles

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**ABBREVIATIONS**

α-Ltx, α-latrotoxin; DIV, days in vitro; DSS, Disuccinimidyl Suberate; ECFP, enhanced cyan fluorescent protein; ER, endoplasmic reticulum; EYFP, enhanced yellow fluorescent protein; FP, fluorescent protein; GFP, green fluorescent protein; GSDB, goat serum dilution buffer; KRH, Krebs-Ringer’s solution; LDL-R, low density lipoprotein receptor; MAP2, microtubule-associated protein 2; PBS, phosphate-buffered saline; PEI 25, 25-kDa polyethylenimine; SLMV, synaptic-like microvesicle; SV, synaptic vesicle; SV2, synaptic vesicle protein 2; SytI, synaptotagmin I; SytI, synaptotagmin I; TfR, transferrin receptor; TGN, trans-Golgi network; VAMP, vesicle-associated membrane protein.
ABSTRACT

Synaptic vesicle (SV) proteins are synthesized at the level of the cell body and transported down the axon in membrane precursors of SVs. To investigate the mechanisms underlying sorting of proteins to SVs, fluorescent chimeras of vesicle-associated membrane protein (VAMP) 2, its highly homologous isoform VAMP1 and synaptotagmin I (SytI) were expressed in hippocampal neurons in culture. Interestingly, the proteins displayed a diffuse component of distribution along the axon. In addition, VAMP2 was found to travel in vesicles which constitutively fuse with the plasma membrane. Co-expression of VAMP2 with synaptophysin I (SypI), a major resident of SVs, restored the correct sorting of VAMP2 to SVs. The effect of SypI on VAMP2 sorting was dose-dependent, being reversed by increasing VAMP2 expression levels, and highly specific, since the sorting of the SV proteins VAMP1 and SytI was not affected by SypI. The cytoplasmic domain of VAMP2 was found to be necessary for both the formation of VAMP2-SypI hetero-dimers and for VAMP2 sorting to SVs. These data support a role for SypI in directing the correct sorting of VAMP2 in neurons, and demonstrate that a direct interaction between the two proteins is required for SypI in order to exert its effect.
INTRODUCTION

Neuronal membrane proteins are subjected to multiple sorting steps: they are sorted to the axonal or somatodendritic compartment and, within the same compartment, they may be incorporated into different organelles.

In the case of SVs, the transport along the axon of “packets” of proteins destined to the synapse has been described (Ahmari et al., 2000; Almenar-Queralt and Goldstein, 2001). The precursor vesicles transported down the axon are morphologically distinct from mature SVs (Tsukita and Ishikawa, 1980), and various types of carriers transporting distinct cargoes exist (Okada et al., 1995; Hirokawa, 1996; Zhai et al., 2001). Thus, mature SVs do not seem to be generated by budding from the trans-Golgi network (TGN), and their assembly is likely to occur at the synapse.

Two lines of evidence suggest that SV proteins might leave the TGN in vesicles of the constitutive secretory pathway. In PC12 cells constitutive secretory vesicles mediate the transport of SypI, a marker of synaptic-like microvesicles (SLMVs), from the TGN to the plasma membrane, in a process which involves fusion with endosomal compartments (Regnier-Vigouroux et al., 1991). Whether this pathway is taken also by other SLMV components and to what extent this model can be applied to neuronal SVs is unknown.

Studies concerning the SV protein VAMP2 have provided evidence for the existence of separate motifs for axonal sorting and for incorporation into SVs, raising the possibility that these are separate steps (Grote et al., 1995; West et al., 1997). Recent work from Banker’s group indicates that accumulation of VAMP2 in the axon is due to retention, rather than to selective sorting, implying a role for molecular interactions which occur exclusively in this compartment (Sampo et al., 2003). When exogenously expressed in cultured hippocampal neurons VAMP2 was shown to display a diffuse component of distribution along the axonal plasma membrane. This distribution was interpreted as either a result of protein spillover from synaptic sites upon SV exocytosis.
(Sankaranarayanan and Ryan, 2000; Li and Murthy, 2001) or lack of a developmentally regulated sorting control system (Ahmari et al., 2000).

Several interactions between pairs of SV proteins have been described. Whereas the importance of such interactions for SV exocytosis is well established, their role in SV biogenesis is obscure. A role for the AP3 adaptor in recruiting VAMP2 during SV budding from the endosomal membrane has been suggested (Salem et al., 1998). In addition, VAMP2 is known to form a complex on the SV membrane with SypI (Calakos and Scheller, 1994; Washbourne et al., 1995; Edelmann et al., 1995; Galli et al., 1996; Pennuto et al., 2002).

A possible function of SypI in SV biogenesis has been hypothesized based on its ability to interact with cholesterol (Thiele et al., 2000). Thus, SypI might be involved in the formation of lipid microdomains where SV membrane constituents are pre-assembled, a situation similar to that observed for the apical transport of proteins in epithelial cells. In addition, the ability of SypI to form oligomers might promote membrane curvature, facilitating the budding of SVs from the donor membrane (Hannah et al., 1999).

To improve our understanding of the processes of SV formation and of the role played by SypI, we have directly visualized fluorescent chimeras of SV proteins in living cells. The results obtained indicate that SypI is required to recruit VAMP2 to SVs, but does not provide a general assembly mechanism for all SV proteins.
MATERIALS AND METHODS

Antibodies

The monoclonal antibody against synaptic vesicle protein 2 (SV2) was provided by Dr. K. Buckley (Harvard University, Boston, MA). Monoclonal antibodies against SypI and VAMP2 and polyclonal antibody against VAMP1 were from Synaptic Systems (Göttingen, Germany). The polyclonal antibody against SypI has been previously described (Valtorta et al., 1988). The monoclonal antibody against microtubule associated protein 2 (MAP2) was from Roche Molecular Biochemicals, (Indianapolis, IN). The 3E6 monoclonal antibody against green fluorescent protein (GFP) was from Quantum Biotechnologies (Montreal, Canada).

DNA constructs

The SypI-EYFP, SypI-ECFP, EYFP-VAMP2, ECFP-VAMP2, SytI-EYFP expressing vectors have been previously described (Pennuto et al., 2002). The VAMP2-GFP expressing vector was a kind gift of Dr. R. Scheller (Stanford University School of Medicine, Stanford, CA). Rat VAMP1 full-length cDNA (357 base pairs) cloned into the pBlue-Script vector (Stratagene, La Jolla, CA) was obtained from Drs. C. Montecucco and O. Roste (University of Padua, Italy). VAMP1 cDNA was amplified by PCR with the following oligonucleotides: forward, 5’GGGGGTGTACAAGATGTCTGCTCCAGCTCAGCC-3’; and reverse, 5’-GGGGGCGGCCGC TCAAGTAAAAATGTAGATTA-3’. BsrGI and NotI restriction sites, introduced by the forward and reverse primers, respectively, are underlined. The resultant BsrGI/NotI PCR fragment was cloned in frame at the carboxy (C)-terminal of ECFP in the corresponding sites of pECFP-N3 vector (Clontech, Palo Alto, CA), generating the pECFP-VAMP1 vector. Human transferrin receptor (hTfR) cDNA (2300 base pairs) cloned into the pCMV5 plasmid was a gift of Dr. D. Zacchetti (San Raffaele Scientific Institute, Milan, Italy). TfR cDNA was extracted by an EcoRI cut and cloned in frame at the C-terminal of EGFP in the corresponding site
of pEGFP-C3 vector (Clontech, Palo Alto, CA). EGFP was substituted by EYFP extracted from pEYFP-N3 vector by a Nhel/BsrGI cut, thus generating the pEYFP-TfR vector. The ECFP-VAMP2/CtVAMP1 and EYFP-VAMP1/CtVAMP2 expressing vectors were produced by swapping the amino (N)-terminal domains of VAMP1 (aa 1-99) and VAMP2 (aa 1-96) extracted by a BsrGI/BclI cut from pECFP-VAMP1 and pEYFP-VAMP2, respectively. A schematic representation of the fluorescent chimeras employed in this study is shown in Figure 1.

**Cell culture and Transfection**

Primary neuronal cultures were prepared from the hippocampi of Sprague-Dawley E18 rat embryos (Charles River Italica, Calco, Italy) as previously described (Banker and Cowan, 1977). Neurons were transfected at 3 days *in vitro* (DIV) using 25-kDa polyethylenimine (PEI 25) (Sigma-Aldrich, Steinheim, Germany) as described by Pennuto et al. (2002). Briefly, PEI (28 nmoles/dish) and plasmid DNA (2.5 µg/dish) were diluted in 50 µl of 150 mM NaCl in separate tubes, mixed and vortexed four times within 12 min. Immediately before transfection, coverslips were placed in a clean 35 mm Petri dish, rinsed with minimal essential medium supplemented with 10% horse serum, 2 mM glutamine, and 3.3 mM glucose and then incubated for 2 h at 37° C in a 5% CO2 humidified atmosphere with 1 ml of the same medium containing the PEI 25/DNA mixture. After incubation the coverslips were returned to the original dishes and maintained in culture until 15 DIV. Living transfected neurons were imaged at room temperature in Krebs-Ringer’s solution (KRH) (150 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2 mM CaCl2, 10 mM glucose, 10 mM HEPES/Na, pH 7.4). For the experiments in which changes in the localization of the fluorescent proteins upon exocytosis were monitored, transfected neurons at 15 DIV were rapidly rinsed with KRH supplemented with 2 mM EGTA (KRH/EGTA) and subsequently incubated for 40 min at 37° C in 5% CO2 in the same solution containing 0.1 nM α-latrotoxin (α-Ltx) (a gift of Dr. A. Petrenko, New York University Medical Center, NY).
Immunofluorescence

Cells to be processed for immunofluorescence were fixed for 30 min with 4% paraformaldehyde, 4% sucrose in 120 mM sodium phosphate buffer (pH 7.4), rinsed with phosphate-buffered saline (PBS) and incubated overnight at 4°C with the primary antibody appropriately diluted in goat serum dilution buffer (GSDB) (15% goat serum, 450 mM NaCl, 0.3% Triton X-100, 20 mM sodium phosphate buffer, pH 7.4). Incubation with the appropriate secondary antibodies (Jackson ImmunoResearch, West Grove, PA) was carried out for 1-2 h at room temperature. Specimens were then washed three times within 30 min with high salt buffer (500 mM NaCl, 20 mM sodium phosphate buffer, pH 7.4) and once with 5 mM sodium phosphate buffer, pH 7.4. Coverslips were mounted with 70% glycerol in PBS supplemented with phenylenediamine (1 mg/ml; Sigma-Aldrich) as an anti-bleaching agent.

For cell surface detection of VAMP2-EGFP, living cells were incubated for 10 min at 37°C with anti-GFP antibody diluted in 10% horse serum, 2 mM glutamine, and 3.3 mM glucose, rinsed briefly in PBS and fixed as described above. Cells were incubated for 1 h at room temperature with tetramethylrhodamine isothiocyanate-conjugated anti-mouse antibody (Jackson ImmunoResearch) in GSDB, washed and mounted as described above.

Videomicroscopy and Quantification

Specimens were viewed with a Zeiss (Oberkochen, Germany) Axiovert 135 inverted microscope equipped with epifluorescence optics. Images were recorded with a C4742-98 ORCA II cooled charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) and processed using Image Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD) and Adobe Photoshop 6.0 (Adobe System, San Jose, CA).

For the quantification of synaptically versus extra-synaptically located protein, an average of 450 synaptic boutons belonging to 6 distinct cells from at least two different experiments were analyzed.
for each condition. Six representative 12 bit (1024×1024 pixels) images of axonal processes were acquired with a 40× oil immersion objective. Acquisition parameters were maintained constant in all experiments. In the case of endogenous VAMP2, FP-VAMP2 coexpressed with cytosolic EYFP and SypI-FP, the staining pattern for endogenous SV2 was used to prepare a binary mask in which each spot corresponded to a synaptic bouton. In all other cases, the synaptic mask was prepared using the distribution pattern of FP-SypI coexpressed in the same cells. Diffusion of the chimeras was expressed as the ratio between the amount of protein (number of pixels × average fluorescence) localized outside and within synaptic boutons (F_{out}/F_{in}).

**Cross-linking analysis**

In order to analyze the formation of SypI and VAMP2 hetero- and homo-complexes, 15 DIV hippocampal neurons were rinsed once with KRH/EGTA and subsequently incubated for 45 min at room temperature in the same solution supplemented with 0.5 mM Disuccinimidyl Suberate (DSS) (Pierce, Rockford, Illinois). At the end of the incubation, TRIS-NaOH (pH 7.4) was added to the final concentration of 100 mM. After 30 min, the neurons were processed for immuno-precipitation with either polyclonal anti-SypI or monoclonal anti-VAMP2 antibodies as previously described (Becher *et al.*, 1999). Gel electrophoresis and immunoblotting were carried out as previously described (Menegon *et al.*, 2002).

In other experiments, SVs purified from rat forebrain through the step of sucrose density gradient (SG2 fraction; Huttner *et al.*, 1983) were subjected to chemical cross-linking with DSS (0.2 mg/ml final concentration) for 1 h at room temperature. The reaction was blocked by the sequential addition of 100 mM glycine and Laemmli stop buffer (Laemmli, 1970) and the samples were subjected to SDS-PAGE and immunoblotting.
RESULTS

Overexpressed VAMP2 is sorted to the axon of hippocampal neurons in culture.

A chimera made by enhanced cyan fluorescent protein (ECFP) fused to the cytosolic N-terminal portion of the SV protein VAMP2 (Figure 1) was expressed in hippocampal neurons in culture, and its targeting was tracked by videomicroscopy imaging of live cells. Transfected neurons were maintained in culture until they acquired full functional maturation (DIV 15; Valtorta and Leoni, 1999). In the axon, the overexpressed fluorescent chimera showed a diffuse pattern of distribution, similar to that displayed by soluble enhanced yellow fluorescent protein (EYFP) transfected in the same cells (compare Figures 2A and A’). However, ECFP-VAMP2 was enriched in puncta which were stained by the endogenous SV markers SV2 and SypI (Bajjalieh et al., 1994; Navone et al., 1986), and could therefore be identified as synaptic boutons (Figures 2B-B’’ and 2C-C’’). Only a small fraction of VAMP2-positive puncta were observed to move in either anterograde or retrograde direction (our unpublished results), identifying them as travelling packets (Ahmari et al., 2000; Nakata et al., 1998; Kaether et al., 2000). In order to assess whether at the level of puncta ECFP-VAMP2 was present in functional SVs, exocytosis was stimulated by α-Ltx (Figure 2D). When applied in the absence of extracellular Ca$^{2+}$, α-Ltx causes massive SV exocytosis, which is not followed by endocytosis (Valtorta et al., 1988). As previously described (Pennuto et al., 2002), α-Ltx-stimulated exocytosis resulted in the formation of two distinct populations of synaptic boutons with different size. Exocytosis-induced insertion of the chimera into the axonal plasma membrane generated a ring of fluorescence at the periphery of the large boutons.

Virtually no colocalization of the VAMP2-positive neurites with the somato-dendritic markers microtubule-associated protein 2 (MAP2; Figure 2F-F’’) and transferrin receptor (TfR; our unpublished results) (Kosik and Finch, 1987; Cameron et al., 1991) was observed, indicating that the chimera is specifically targeted to the axon of transfected cells. Only when the expression levels
of ECFP-VAMP2 were exceedingly high the chimeric protein was found also in dendrites (not shown). These cells, which exhibited signs of toxicity, were excluded from all subsequent analyses. To determine the proportion of ECFP-VAMP2 that diffused along the axon with respect to the amount that was localized at the level of puncta, the fluorescence intensity and number of VAMP2-positive pixels which colocalized with endogenous SV2 was quantified and compared with the intensity and number of those that did not colocalize with SV2. This analysis indicated that the bulk of ECFP-VAMP2 was located outside synaptic boutons (Figure 3). A similar analysis was performed for endogenous VAMP2 revealed by indirect immunofluorescence. In this case, the majority of VAMP2 appeared to be confined to synaptic boutons (Figures 2E and 3). The lack of diffusion of endogenous VAMP2 in both immature (DIV 3; our unpublished results) as well as mature (DIV 15) neurons grown under similar conditions suggests that the diffuse distribution of ECFP-VAMP2 is the result of its overexpression in transfected cells. To rule out the possibility that the diffusion of ECFP-VAMP2 was due to the presence of the fluorescent protein fused to the cytosolic N-terminal portion of VAMP2, we analyzed the distribution of a chimera in which GFP is fused to the intraluminal C-terminal portion of VAMP2 (Figure 1). The VAMP2-GFP chimera, transfected in hippocampal neurons, showed a diffuse pattern of fluorescence similar to that displayed by ECFP-VAMP2 (Figure 4A, compare with Figure 2A). Quantification of the diffusion of VAMP2-GFP with respect to endogenous SV markers gave results similar to those obtained for ECFP-VAMP2 (Figure 3).

**Exogenous VAMP2 is present in vesicles which constitutively fuse with the axonal plasma membrane**

The possibility that the extrasynaptic VAMP2 chimera was localized on the plasma membrane was tested by applying anti-GFP antibodies to live, unfixed neurons transfected with VAMP2-GFP. In these cells, the staining pattern determined in the axon by the anti-GFP antibody was virtually identical to that of VAMP2-GFP, indicating that exogenous VAMP2 is actually exposed to the
extracellular surface of the plasma membrane along the axon of transfected cells (Figure 4A and A’). As expected, the anti-GFP antibody could not stain the chimera in the Golgi complex (Figure 4B and B’).

**SypI directs the sorting of VAMP2 to synapses**

Since SypI directly interacts with VAMP2 and has been involved in the process of SV biogenesis, we investigated whether the protein might affect VAMP2 sorting in hippocampal neurons. Interestingly, the chimera SypI-EYFP, in which EYFP is fused to the cytosolic C-terminal tail of SypI (Figure 1), always appeared to be selectively localized at synaptic boutons, and was never observed to diffuse along the axonal membrane, independently of the level of expression of the protein or the developmental stage of the cells (Figures 3 and 5A’-C’). These results are in agreement with previous reports of localization of the chimera into functional SVs (Pennuto et al., 2002).

SypI-EYFP was co-transfected in hippocampal neurons together with ECFP-VAMP2 using various ratios of expression plasmids for the two proteins. When ECFP-VAMP2 and SypI-EYFP were cotransfected in a 1:4 ratio (Figures 5A-A’), exogenous VAMP2 showed a well defined punctated distribution, with very low levels of protein diffused outside the puncta (Figure 3). ECFP-VAMP2 positive puncta precisely coincided with SypI-EYFP puncta and with endogenous SV2, defining them as synapses. Similar results were obtained when ECFP-VAMP2 and SypI-EYFP expression plasmids were cotransfected in a 1:1 ratio (Figures 3 and 5B-B’’).

In contrast, cotransfection of ECFP-VAMP2 and SypI-EYFP in a 4:1 ratio led to a clear diffusion of ECFP-VAMP2 along the axons, a pattern virtually indistinguishable from that observed when the chimera was expressed in the absence of exogenous SypI (Figures 5C-C’’, compare with Figure 2A). Indeed, in this case the proportion of diffused VAMP2 was similar to that observed for VAMP2 overexpressed in the absence of SypI (Figure 3). The lowest amount of ECFP-VAMP2
The plasmid used in the cotransfection experiments corresponded to that used in the previous experiments (i.e. an amount sufficient to lead to diffusion of the chimera along the axons).

In order to assess the specificity of the effect of SypI on VAMP2 sorting, EYFP was fused to the cytosolic C-terminal tail of the single-pass SV protein synaptotagmin I (SytI), to generate the SytI-EYFP chimera (Figure 1). When neurons were co-transfected with the expression vectors for ECFP-VAMP2 and SytI-EYFP in a 1:1 ratio, both chimeras displayed a diffuse pattern of distribution along the axons (Figures 5D-D’’), although they were enriched at the level of synaptic sites marked by antibodies to endogenous SV2 (our unpublished results).

**The expression of SypI does not affect sorting of proteins destined to the somato-dendritic compartment.**

The possibility that overexpressed SypI might alter polarized membrane trafficking in neurons was tested by analyzing the distribution of the somatodendritic protein TfR in neurons overexpressing SypI. The EYFP-TfR chimera, in which the TfR N-terminal end was fused to EYFP (Figure 1), was co-expressed in hippocampal neurons together with SypI-ECFP. Neurons were cotransfected with the expression plasmids for EYFP-TfR and SypI-ECFP in a 1:4 ratio.

The two chimeras colocalized in the Golgi complex and were then specifically sorted to their correct subcellular compartments. Thus, SypI-ECFP was exclusively present at synaptic sites along the MAP2-negative axon, whereas EYFP-TfR was localized in the MAP2-positive somatodendritic compartment of the transfected neurons (Figure 6).

**Sorting of the SV proteins SytI and VAMP1 does not depend on SypI.**

The dependence of VAMP2 sorting on SypI expression prompted us to investigate whether SypI also regulates the sorting of other SV proteins. In order to explore this possibility, we studied the distribution of SytI-EYFP in hippocampal neurons expressing SypI-ECFP. When SytI-EYFP was overexpressed together with SypI-ECFP in either a 1:1 or a 1:4 ratio, it showed a diffuse pattern of
distribution (Figure 7A and A’, and our unpublished results), similar to that displayed by SytI-EYFP when overexpressed together with either ECFP-VAMP2 (Figure 5D and D’) or with soluble ECFP (our unpublished results). The pattern of distribution of SytI-EYFP was reminiscent of that of ECFP-VAMP2 expressed in the absence of SypI (Figures 2 and 3). Similarly to the VAMP2 chimera, SytI-ECFP was enriched at synaptic sites, identified by both exogenous SypI-ECFP and endogenous SV2. Upon treatment with $\alpha$-Ltx, SytI-ECFP present in puncta was translocated to the plasma membrane as a result of exocytosis, thus indicating that a certain amount of the exogenous protein is delivered to functional SVs (our unpublished results).

We next studied whether SypI could control the sorting of VAMP1, a VAMP family member highly homologous to VAMP2 and expressed on SVs. The cytosolic N-terminal portion of VAMP1 was fused to ECFP (Figure 1), and the resulting chimera (ECFP-VAMP1) was overexpressed in neurons together with soluble EYFP. ECFP-VAMP1 showed a diffuse pattern of localization along the axons, with enrichments at the level of synaptic sites stained by SV2 (our unpublished results). A virtually identical pattern of distribution was detected when ECFP-VAMP1 was transfected in neurons together with SypI-EYFP in a 1:1 ratio (Figure 7B and B’).

**The amino-terminal portion of VAMP2 is required for the interaction with SypI.**

The specificity of the SV targeting effect of SypI for VAMP2 is likely to depend on the formation of VAMP2-SypI heterodimers previously reported to occur in SVs from brain homogenates and hypothesized to play a regulatory role in exocytosis (Washbourne *et al.*, 1995). The specificity of this interaction was tested in SVs purified from rat brain and cross-linked by treatment with DSS. In these samples, both SypI and VAMP2 homo-dimers as well as SypI-VAMP2 hetero-dimers could be visualized, whereas SypI-VAMP1 heterodimers were not detected (Figure 8A). The formation of the VAMP2-SypI complex was detected biochemically also in cultured hippocampal neurons after protein cross-linking followed by immunoprecipitation with either anti-VAMP2 or anti-SypI antibodies and immunoblotting (Figure 8B).
We examined in live cells whether the cytosolic N-terminal region of VAMP2, but not that of VAMP1, is required for the control of VAMP2 sorting by SypI. To this purpose, we swapped the N-terminal domains of VAMP2 and VAMP1 and fused the resulting constructs to ECFP and EYFP, generating the ECFP-VAMP2/CtVAMP1 and EYFP-VAMP1/CtVAMP2 chimeras (Figure 1). As expected, when transfected in hippocampal neurons together with soluble EYFP or ECFP, the two chimeras showed a diffuse pattern of distribution (our unpublished results). When ECFP-VAMP2/CtVAMP1 or EYFP-VAMP1/CtVAMP2 were coexpressed in neurons together with either SypI-EYFP or SypI-ECFP respectively, ECFP-VAMP2/CtVAMP1 displayed a synaptic distribution, whereas EYFP-VAMP1/CtVAMP2 showed a diffused distribution (Figure 8C), indicating that the N-terminal portion of VAMP2 is necessary for its recruitment to synaptic sites by SypI.
DISCUSSION

The molecular sorting events which lead to the formation of mature SVs are still poorly understood. In an attempt to clarify this issue, we took advantage of the use of spectrally separated variants of GFP fused in frame with various SV proteins to directly observe the fate of the resulting fluorescent chimeras in live hippocampal neurons.

The fluorescent chimeras of all the SV proteins tested were largely confined to the axon, indicating that they contain axonal targeting information. However, whereas Sypl was selectively confined to SVs, the other SV proteins, namely SytI, VAMP2 and VAMP1 when overexpressed were not exclusively localized to synaptic sites. Rather, they were diffuse all over the surface of the axonal plasma membrane, albeit some enrichment at the level of synaptic puncta could be detected. Thus, although these proteins bear the information to be sorted to the axon, they require additional signals to be recruited to SVs.

Because of its ability to bind to both lipidic and proteinaceous components of SVs, Sypl has been proposed to play a key role in the biogenesis of this organelle (Thiele et al., 2000). Thus, Sypl appeared to be a potential candidate for rescuing the correct targeting of the other SV proteins. Interestingly, Sypl was selectively able to recruit axonal VAMP2 to SVs. The localization of both proteins on functional SVs was indicated by their colocalization with endogenous SV markers and by the redistribution of the fluorescent signal to the plasma membrane of puncta upon α-Ltx-stimulated exocytosis (Pennuto et al., 2002). The effect of Sypl on VAMP2 was dose-dependent, and correct targeting of VAMP2 to SVs was achieved when the range of expression of the exogenous proteins was similar to that of the endogenous proteins. On the other hand, neither VAMP1 nor SytI could be recruited to SVs even at very high Sypl expression levels.

The fact that Sypl is unable to redirect the sorting of VAMP1, although the latter is highly homologous to VAMP2, suggests that the effect of Sypl on VAMP2 sorting requires a direct interaction between the two proteins. Indeed, at mature synapses Sypl is known to interact with
VAMP2, whereas the possibility of a direct interaction with VAMP1 is controversial (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995; Bacci et al., 2001). As far as SytI is concerned, the existence of a direct interaction between SytI and SypI on the SV membrane has been excluded (Pennuto et al., 2002), although the two proteins have been shown to be transported along the axon in the same carrier vesicle (Okada et al., 1995).

Overall, VAMP1 and VAMP2 sequences show a high degree of homology. Interestingly, they maximally diverge in their proline-rich amino-terminal portions, which in the case of VAMP2 has been shown to be required for its interaction with SypI (Washbourne et al., 1995; Bacci et al., 2001). Consistently, when the amino-terminal region of VAMP2 was substituted with that of VAMP1, the resultant protein diffused in the presence as well as the absence of SypI. Vice versa, when the amino-terminal region of VAMP1 was replaced by that of VAMP2, the chimera was seen to diffuse when expressed in the absence of SypI, whereas it concentrated at synaptic sites when expressed in the presence of SypI. These findings indicate that the cytosolic tail of VAMP2, which is responsible for its interaction with SypI, is also necessary for its SypI-mediated recruitment to SVs. It has previously been reported that the cytosolic tail of VAMP2 contains a sequence which negatively regulates its targeting to SVs (West et al., 1997). Our results are consistent with the hypothesis that SypI, by interacting with VAMP2, leads to the masking of this negative regulator, thus allowing the retention of VAMP2 in SVs.

Two possibilities must be taken into account: SypI might specifically recruit some newly synthesized SV components at the level of the TGN or it might recruit SV components directly at the nerve terminal, from either the plasma membrane or endosomes. Although the possibility of the formation of SVs from the TGN cannot be formally excluded, in the past few years several lines of experimental evidence have accumulated suggesting that SV proteins are transported along the axon in precursor vesicles which are assembled into mature SVs in the presynaptic nerve terminal (see, e.g., Hirokawa, 1998).
In the perikarion VAMP2 has been shown to codistribute with SytI, p29 and SV2, but not with SypI (Mundigl et al., 1993). In addition, it has recently been reported that VAMP2 is delivered to both axons and dendrites, but is preferentially endocytosed from the dendritic membrane. Thus, its polarized distribution to the axonal compartment can be ascribed to selective retention, rather than to selective delivery (Sampo et al., 2003).

SypI is unlikely to be involved in mediating the selective retention of VAMP2 in the axonal compartment, since the latter accumulates in the axonal membrane also when overexpressed in the absence of stoichiometric amounts of SypI. However, the SypI-VAMP2 interaction appears to be necessary for the recruiting of VAMP2 to SVs, either by inducing endocytosis of VAMP2 from the axonal plasma membrane or by facilitating sorting from the endosomal compartment.

In the PC12 neuroendocrine cell line, SypI has been shown to exit the TGN in constitutive vesicles, and to undergo at least one cycle of fusion with the plasma membrane and recycling through endosomes before being incorporated into SLMVs (Regnier-Vigouroux et al., 1991). If this behaviour can be extrapolated to neurons, then it is possible that the interaction of SypI with VAMP2 at some step of the recycling process leads to the recruitment of the latter to SVs.

Under conditions in which exocytosis is stimulated in the absence of endocytosis VAMP2 was found to dissociate from SypI prior to fusion of the vesicles with the plasma membrane (Pennuto et al., 2002). However, when exocytosis is balanced by compensatory levels of endocytosis, SypI and other SV markers do not accumulate in the presynaptic plasma membrane (Valtorta et al., 1988; Torri-Tarelli et al., 1990 and 1992).

Endocytosis seems to be a saturable mechanism of SV retrieval which slows with stimulus increase, giving rise to the diffusion of SV proteins along the plasma membrane away from the sites of exocytosis (Sankaranarayanan and Ryan, 2000). Recently, SypI and VAMP2 have been shown to be recovered to SVs with similar kinetics after exocytosis, suggesting a similar mechanism of recovery for the two proteins during recycling (Li and Murthy, 2001). These findings suggest that the diffusion of some SV proteins along the axonal plasma membrane observed in this study might
result from the saturation of the machinery involved in the recruitment of such proteins to SVs, and support a model in which SypI might play a pivotal role in directing VAMP2 targeting to SVs.

The apparent discrepancy between our results and the lack of phenotype in mice deleted for the sypI gene (Eshkind and Leube, 1995; McMahon et al., 1996) might be due to compensatory effects exerted by other members of the synaptophysin family (see, e.g. Spiwoks-Becker et al., 2001; Janz et al., 1999). Indeed, a role for SypI in activity-dependent synapse formation has been highlighted by the use of heterogenotypic cocultures of neurons from wild type and knock-out mice (Tarsa and Goda, 2002).

In conclusion, the present work shows that at least four SV proteins (SypI, VAMP2, VAMP1, and SytI) bear the targeting domains for a polarized sorting to the axon. SypI retains the ability to be exclusively confined to SVs, while the other three SV proteins investigated are recruited to both regulated and constitutive secretory vesicles, indicating that the proteins require additional signals to be correctly sorted to SVs. Protein-protein interaction is at the basis of the ability of VAMP2 to be recruited to SVs by SypI. Further work will be required to identify the protein-protein interactions affecting the sorting of VAMP1 and SytI.
REFERENCES


FIGURE LEGENDS

Figure 1. Schematic representation of the employed fluorescent chimeras.
The SV proteins VAMP2, VAMP1, SytI and SypI, as well as TfR were fused to either enhanced cyan, yellow, or green fluorescent proteins (FP), here represented by white boxes. ECFP-VAMP2/CtVAMP1 and EYFP-VAMP1/CtVAMP2 chimeras were produced by swapping the N-terminal regions of VAMP1 and VAMP2 fused to ECFP and EYFP, respectively. The orientation of the proteins in the membrane lipid bilayer is indicated.

Figure 2. Overexpression leads to ECFP-VAMP2 mis-sorting along the axon of transfected hippocampal neurons.
(A and A’) 15 DIV hippocampal neurons co-expressing ECFP-VAMP2 (A) and cytosolic EYFP (A’). ECFP-VAMP2 overexpression results in a diffuse distribution of the protein along the axon of the transfected cells, although the protein is enriched in puncta. Some of these puncta show also enhanced staining for soluble EYFP and probably represent sites of increased thickness of the axon. (B-B’’; C-C’’) ECFP-VAMP2 (B and C) colocalizes in puncta with the endogenous SV markers SV2 (B’), and SypI (C’). (B’’ and C’’’) Merge of previous images. ECFP-VAMP2 is shown in green, SV2 and SypI are in red.

(D) Neurons expressing ECFP-VAMP2 treated for 30 min with 0.1 nM α-Ltx. The chimera is present in functional synapses, which undergo swelling because of the massive toxin-stimulated exocytosis. The incorporation of ECFP-VAMP2 into the plasma membrane after exocytosis is apparent after focussing on the surface of a large bouton (inset). No major changes in the appearance of extra-synaptic ECFP-VAMP2 are visible upon α-Ltx stimulation.

(E) Distribution of endogenous VAMP2 in hippocampal neurons at 15 DIV.
(F-F’’) Neurons expressing ECFP-VAMP2 (F) and stained for the somatodendritic marker MAP2 (F’). (F’’) Merge of the previous images. ECFP-VAMP2 is shown in green, MAP2 in red. ECFP-VAMP2 distribution is mainly polarized to the axons.

Bar, 10 μm for A-C’’, 17 μm for E and F-F’’, 6 μm for D, 3 μm for the inset in D.

**Figure 3. Quantification of the distribution of the SV fluorescent proteins.**

The ratio \( \frac{F_{\text{out}}}{F_{\text{in}}} \) (± S.D.) between the amount of exogenous fluorescent SV proteins present outside and inside synaptic boutons was calculated and compared with the distribution of endogenous VAMP2. For each experimental condition, the protein analyzed is underlined.

**Figure 4. Exogenous VAMP2 is present on the axonal plasma membrane.**

Hippocampal neurons (15 DIV) expressing the VAMP2-GFP chimera, in which GFP is fused to the intravesicular domain of the protein.

(A) VAMP2-GFP diffuses along the axon of transfected cells. (A’) Surface staining of live unfixed neurons with an anti-GFP antibody. (B-B’) Cell body from the same transfected neuron. Due to membrane integrity, VAMP2-GFP in the Golgi compartment (B) is not accessible to the anti-GFP antibody (B’).

Bar, 10 μm.

**Figure 5. SypI corrects the mis-sorting of exogenous VAMP2**

Hippocampal neurons (15 DIV) co-transfected with ECFP-VAMP2 (A-D) and either SypI-EYFP (A’-C’) or SytI-EYFP (D’). For each condition the ratio of transfected plamids is shown on the left. The expression plasmids for ECFP-VAMP2 and SypI-EYFP were transfected in a ratio of either 1:4 (A-A’), 1:1 (B-B’), or 4:1 (C-C’). SypI exerts a dose-dependent rescue of missorted VAMP2 to its correct synaptic localization. Inset in (A), co-localization of ECFP-VAMP2 (green) and endogenous SV2 (red).
(D-D’) The expression plasmids for ECFP-VAMP2 and SytI-EYFP were co-transfected in a 1:1 ratio. Both chimeras diffuse along the axons of transfected neurons.

(A’’-D’’) Merge of previous images. ECFP-VAMP2 is shown in green, SytI-EYFP (A’’-C’’) and SytI-EYFP (D’’) are in red.

Bar, 10 µm.

**Figure 6. Polarized trafficking of TfR to the somatodendritic compartment is not altered by SypI overexpression.**

Hippocampal neurons (15 DIV) co-transfected with the expression plasmids for EYFP-TfR and SypI-ECFP in a 1:4 ratio. (A) SypI-ECFP is visible at the level of the Golgi complex and in synaptic boutons along the axon. (B) EYFP-TfR colocalizes with SypI-ECFP in the Golgi complex and is trafficked to the somato-dendritic compartment, labeled by an anti-MAP2 antibody (C). (D) Merge of (A) and (B): SypI-ECFP is shown in green, EYFP-TfR in red.

Bar, 10 µm.

**Figure 7. The sorting of SytI and VAMP1 is unaffected by overexpression of SypI**

Hippocampal neurons (15 DIV) co-transfected with the expression vectors for SypI-EYFP (A’-B’’) and either SytI-ECFP (A) or ECFP-VAMP1 (B) in a 1:1 ratio. Both SytI-ECFP (A) and ECFP-VAMP1 (B) show a diffuse extra-synaptic distribution along the axon of transfected cells, although they appear enriched at the level of synaptic puncta, where SypI-EYFP is concentrated.

Bar, 10 µm.

**Figure 8. The interaction of SypI with the cytoplasmic tail of VAMP2 is required for the synaptic sorting of VAMP2.**

(A) Purified SVs from rat brain were treated with the cross-linker DSS where indicated. Protein extracts were analyzed by Western Blotting and probed with anti-SypI and either anti-VAMP2 or
anti-VAMP1 antibodies. Both SypI and VAMP2 homo-dimers and SypI-VAMP2 hetero-oligomers are visible. Virtually no SypI-VAMP1 hetero-dimers are detected. (B) SypI and VAMP2 interact with each other in mature hippocampal neurons. Cells were treated with the cross-linker DSS and processed for immunoprecipitation with either anti-SypI or anti-VAMP2 antibodies. Western Blotting with anti-SypI antibodies reveals the presence of SypI-VAMP2 heterodimers as well as SypI monomers and dimers. (C) ECFP-VAMP2/CtVAMP1 (a) and EYFP-VAMP1/CtVAMP2 (c) were transfected together with either SypI-EYFP (b) or SypI-ECFP (d) in a 1:1 ratio. While ECFP-VAMP2/CtVAMP1 shows a synaptic distribution, EYFP-VAMP1/CtVAMP2 displays a diffuse extra-synaptic distribution.

Bar, 10 µm.