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# **Synaptophysin I selectively specifies the exocytic pathway of Synaptobrevin2/VAMP2**

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## SYNOPSIS

Biogenesis and recycling of synaptic vesicles are accompanied by sorting processes that preserve the molecular composition of the compartments involved. In this study we address the targeting of synaptobrevin2/vesicle associated membrane protein 2 (VAMP2), a critical component of the synaptic vesicle fusion machinery, in a heterotypic context where its sorting is not confounded by the presence of other neuron-specific molecules. Ectopically-expressed synaptophysin I interacts with VAMP2 and converts its default surface targeting to a prominent vesicular distribution, with no effects on the targeting of other membrane proteins. Protein-protein interaction is not sufficient for the control of VAMP2 sorting, which is mediated by the carboxy-terminal domain of synaptophysin I. Synaptophysin I directs the sorting of VAMP2 to vesicles prior to surface delivery, without influencing VAMP2 endocytosis. Consistently, dynamin and alpha-SNAP mutants which block trafficking at the plasma membrane do not occlude the effect of synaptophysin I on VAMP2 sorting. These results indicate that the sorting determinants of synaptic vesicle proteins can operate independently of a neuronal context and implicate the association of VAMP2 with synaptophysin I in the specification of the pathway of synaptic vesicle biogenesis.

## INTRODUCTION

Communication in the nervous system relies on exocytosis of neurotransmitter-filled synaptic vesicles (SVs) that release their content at synapses. SVs are locally regenerated in the nerve terminals following exocytosis, whereas *de novo* formation of SVs requires delivery of new constituents from the cell body to synapses. Collapse of SVs with the plasma membrane during exocytosis is followed by endocytosis through clathrin-coated vesicles which fuse with endosomal compartments, from which SVs are regenerated [1]. Thus, segregation of SV proteins from either plasma membrane or endosomal resident proteins needs to take place in order to preserve the molecular identity of SVs [1,2]

Since at least some of the SV proteins are delivered to synapses in distinct membrane carriers, assembly of SVs might proceed through the stepwise sorting of individual or groups of components onto the nascent organelle at the level of either endosomal compartments or the plasmalemma [1]. Importantly, SV proteins do not display common motifs that dictate their trafficking during SV biogenesis and upon expression in non-neuronal cells they segregate in distinct subcellular compartments [3].

The complexity of targeting information of SV proteins prompted the question as to whether a precise hierarchy of sorting determinants could operate during assembly of SVs. Although the molecular mechanisms underlying the sorting of SV proteins from resident components of the donor membranes are as yet unclear, protein-protein and protein-lipid interactions are likely to play a key role in these processes [1,2]. Thus, the sorting information might be present in a subset of SV proteins with the other components being secondarily targeted to SVs by association with the former proteins.

Owing to the essential function of the v-SNARE (vesicle soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) synaptobrevin2/VAMP2 in SV fusion [4, 5] and retrieval [6] the sorting of this protein to SVs has been extensively investigated. Endocytosis and sorting from endosomal compartments are required for axonal polarization and targeting to SVs of VAMP2 [7-10]. Targeting of VAMP2 to presynaptic sites is controlled by synaptophysin I, a major tetraspan SV membrane protein [11, 12]. The role of synaptophysin I in the SV life cycle is still unclear, mainly because of the absence of major phenotypic changes in synaptophysin I

deletion mutants [13-15], although a contribution of the protein to neurotransmitter release, synapse formation and SV endocytosis has been proposed [12].

On the SV membrane VAMP2 is engaged in a complex with synaptophysin I, which is mutually exclusive with the formation of fusogenic SNARE complexes [16-18]. Consistently, stimulation-induced release of VAMP2 from synaptophysin I precedes SV fusion and makes SVs competent for exocytosis [19-22]. The formation of hetero-complexes also underlies the ability of synaptophysin I to regulate the targeting of VAMP2 to SVs [11].

In order to define at which step of the exo-endocytic pathway synaptophysin I recruits newly synthesized VAMP2 to SV precursors and to determine the minimal requirements for the ability of synaptophysin I to direct the sorting of VAMP2, we reconstituted this process in non-neuronal cells allowing us to investigate the intrinsic determinants of VAMP2 sorting in the absence of confounding effects from other neuron-specific molecules. We show that, at early stages along the secretory pathway, synaptophysin I directs sorting of VAMP2 to vesicles exhibiting limited availability for constitutive exocytosis.

## MATERIALS AND METHODS

### Materials

The following primary antibodies were used: mouse anti-synaptophysin I, anti-VAMP2, anti- $\alpha/\beta$ SNAP (Synaptic Systems, Göttingen, Germany); mouse anti-green fluorescent protein (GFP) (for western blotting) and mouse anti-hemagglutinin (HA) (Roche Diagnostics, Indianapolis, IN); mouse anti-GFP (3E6) (for immunofluorescence) (Quantum Biotechnologies, Montreal, Canada) [both anti-GFP (anti-FP) antibodies recognize the spectral variants yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP)]; mouse anti-transferrin receptor (TfR) (Zymed, San Francisco, CA). Texas Red and FITC-conjugated secondary antibodies and pure rabbit IgG antibodies were from Jackson ImmunoResearch (West Grove, PA). Peroxidase-conjugated anti-rabbit was from Bio-Rad (Hercules, CA). The enhanced chemiluminescence detection system was from Amersham Bioscience (Little Chalfont Buckinghamshire, UK) and BCA protein assay reagent was from Pierce Chemical (Rockford, IL). FITC-conjugated transferrin were from Molecular Probes (Eugene, OR).

### DNA constructs

Expression plasmids for enhanced CFP (ECFP), enhanced YFP (EYFP), SypI-CFP, SypI-YFP, SypI $\Delta$ C-CFP, Syt-YFP have been previously described [20, 23]. To generate the pVAMP2-EYFP vector the VAMP2 cDNA was amplified by PCR from the pECFP-VAMP2 vector [20] with the following oligonucleotides: forward, 5'-GGGGCTCGAGATGTCGGCTACCGCTGCC-3' and reverse, 5'-GGGGAAGCTTAGTGCTGAAGTAAACGATGATG-3'. The PCR product was sequenced prior to digestion with XhoI/HindIII (restriction sites underlined) and cloned into the pEYFP-N1 plasmid (Clontech Laboratories, Inc., Palo Alto, CA). The syntaxin13-CFP expression vector was produced by cloning the syntaxin 13 cDNA (kindly provided by Dr. V. Faundez, Emory University, Atlanta, GA) in frame at the C-terminal of ECFP in the pECFP-N3 vector (Clontech). The pEGFP-Rab11 and pEGFP-Rab5 vectors were kind gifts of Drs. M. Zerial (Max Planck Institute, Dresden, Germany) and C. Bucci (Università degli Studi, Lecce, Italy), respectively. EGFP was substituted with ECFP by a NheI/BsrGI cut. The expression vector for farnesylated ECFP was generated from the pEGFP-F plasmid (Clontech) by substitution of

EGFP with ECFP by a NheI/BsrGI cut. The expression plasmids for HA-tagged dynamin and dynamin K44A were a kind gift of Dr. S.L. Schmid (Scripps Research Institute, La Jolla, CA). The expression plasmids for  $\alpha$ -SNAP wild type and L294A mutant were a kind gift of Dr. R.D. Burgoyne (University of Liverpool, Liverpool, UK).

### Cell culture and transfections

Transformed human epithelial cells, HeLa cells, were grown on either plastic dishes or glass coverslips at 37°C in a 5% CO<sub>2</sub> humidified atmosphere in Dulbecco's modified Eagle's medium (Biowhittaker, Verviers, Belgium), supplemented with 10% fetal calf serum (Hyclone, Logan UT, USA), 1% L-glutamine and 100 U/ml penicillin/streptomycin (Biowhittaker).

For immunofluorescence studies, cells grown on glass coverslips were transfected with 1.5-3  $\mu$ g of DNA using a standard Ca<sup>2+</sup>-phosphate precipitation protocol [24]. Cells were analysed 48-72 hours later. For biochemical studies, cells grown on 6 cm plastic dishes were transfected with Lipofectamine2000 (Stratagene, La Jolla CA) according to the manufacturer's instructions, using 2-4  $\mu$ g of each plasmid and 1.6  $\mu$ l of Lipofectamine2000 per  $\mu$ g of plasmid. Transfected cells were analysed 24 hours later. In most of the experiments plasmids were co-transfected at equimolar ratio unless differently indicated. In order to widen the range of expression levels in the experiments shown in Fig. 4C, we pooled data from parallel samples co-transfected with plasmids in a ratio of 1:1 and 1:3.

### Cell labeling protocols

Immunofluorescence experiments were carried out as previously described [25]. For cell surface detection of fluorescent chimeras cells were incubated for 30 minutes at 4°C in the presence of 0.01  $\mu$ g/ $\mu$ l of the 3E6 anti-GFP antibody diluted in either culture medium or Krebs-Ringer's-Hepes solution (KRH; in mM: 150 NaCl, 5 KCl, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 2 CaCl<sub>2</sub>, 10 glucose, and 10 HEPES/Na, pH 7.4). Specimens were washed three times by complete medium substitution with cold KRH during 3 minutes, fixed and processed for immunofluorescence with Texas Red-conjugated anti-mouse antibody in the absence of detergent. For the VAMP2-YFP endocytosis assay, after surface-labeling with anti-GFP antibody at 4°C, cells were washed with KRH and incubated at 37°C for varying periods. After incubation cells were subjected to acid-stripping with a cold solution containing 500 mM NaCl and 200 mM Na-acetate buffer (pH 3)

applied twice for 2 minutes with a washing in cold KRH for 2 minutes in-between in order to remove surface-associated antibody prior to fixation and immunofluorescence analysis. In some cases, surface staining was carried out on fixed specimens, incubated with anti-GFP antibody in the absence of detergent and processed as described above.

For the transferrin uptake assay, transfected cells were serum starved for 1 hour in the presence of BSA (2 mg/ml) prior to incubation for 30 minutes at 37°C in the presence of FITC-conjugated transferrin (30 µg/ml). After incubation, cells were subjected to acid-stripping at 4°C as described above to remove surface-associated transferrin prior to fixation.

### **Immunoprecipitation**

Transfected cells were rinsed twice with cold phosphate-buffered saline (PBS), and harvested by scraping with PBS followed by centrifugation for 3 minutes at 1000 × g. Extraction was performed with 200 µl of extraction buffer (KCl 140 mM, EDTA 2 mM, Hepes-NaOH 10 mM (pH 7.4), 1% (v/v) Triton X-100) supplemented with protease inhibitor cocktail (Sigma-Aldrich) for 1 hour at 4°C under rotation, followed by two sequential centrifugation steps for 5 minutes at 1000 × g and 10 minutes at 20,000 × g. GammaBind™ G-sepharose beads (Amersham Biosciences, Uppsala, Sweden) were washed in extraction buffer and incubated in the presence of 2.6 µg of anti-HA monoclonal antibody for 2 hours at 4°C under rotation. The beads were incubated overnight at 4°C with a volume of total cell extract containing 400 µg of proteins under rotation in the presence of protease inhibitors. The beads were recovered by centrifugation, washed in extraction buffer, resuspended in sample buffer, boiled and subjected to SDS-PAGE and immunoblotting as previously described [25]. The supernatants from the immunoprecipitation procedure were analyzed in parallel.

### **Biotinylation assays**

Cells were cooled at 4°C for 10 minutes in culture medium, rinsed twice with cold KRH and incubated on ice under rocking for 30 minutes with 0.75 mg/ml of cleavable EZ-Link Sulfo-NHS-SS-Biotin (Pierce Chemical). Unreacted biotin was quenched by washing the cells three times during 10 minutes with ice-cold 50 mM Tris-HCl (pH 8) diluted in KRH. For determining surface expression, biotin-labeled cells were lysed as described above. For the endocytosis assay, biotin-labeled cells were incubated at 37°C for various times. Parallel samples left at 4°C to block



membrane trafficking were used as negative controls. After incubation, biotin remaining at the cell surface was removed by reducing the disulfide linkage with three 15-minute incubations with ice-cold sodium 2-mercaptoethanesulfonic acid (MESNA) cleavage buffer (100 mM MESNA, 50 mM Tris-HCl pH 8.6, 100 mM NaCl, 1 mM EDTA, 0.2% BSA) at 4°C. Residual MESNA was quenched by washing the cells three times during 10 minutes with ice-cold 120 mM iodoacetamide diluted in KRH before cell lysis. After centrifugation at 13,000 rpm for 10 minutes, biotinylated proteins were isolated by incubation of 70 µg of cell extracts with UltraLink immobilized streptavidin (Pierce Chemical) for 5-10 hours at 4°C under rotation in the presence of protease inhibitors. The beads were recovered by centrifugation, washed in extraction buffer, resuspended in sample buffer and subjected to SDS-PAGE and immunoblotting. Total cell extracts were analysed in parallel.

### **Videomicroscopy and image analysis**

For live imaging cells were incubated in KRH and maintained at either room temperature or 37°C. 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), Adobe Photoshop 6.0 (Adobe System, San Jose, CA) and the public domain program ImageJ (developed at the US National Institute of Health, Bethesda, Maryland). Prism (GraphPad Software, Inc., San Diego, CA) was used for statistical analysis. Acquisition parameters were maintained constant throughout any single experiment. For measuring colocalization, merged images were modified to the same background and three 6.4 µm<sup>2</sup> square-regions were selected for each cell. Overlap coefficient was calculated for this set of images using a dedicated command of Image Pro Plus 4.5. The number of VAMP2-positive puncta was determined in the same set of images by performing granulometric filtering with the Gran filter plugin of ImageJ followed by automatic counting of particles selected with an appropriate threshold command which was maintained constant for all measurements. For measuring surface to total chimera localization, images were modified to the same background and the ratio between the intensity (number of pixels × average intensity) of anti-FP immunoreactivity to total intrinsic chimera fluorescence was determined in

individual cells. Film densitometry was carried out using dedicated commands of ImageJ. The same background level was subtracted to all lanes.

## RESULTS

### **VAMP2 is primarily targeted to the plasma membrane in non-neuronal cells**

To establish an assay for the sorting determinants of VAMP2, a fluorescent chimera in which EYFP was fused to the intraluminal domain of VAMP2 (VAMP2-YFP) was expressed in HeLa cells that do not express neuron-specific molecules. The patterns of fluorescence of VAMP2-YFP and of a variant of ECFP bearing a farnesylation signal (CFP-F) which directs the protein to the plasma membrane were largely superimposable, indicating that exogenous VAMP2 was predominantly directed to the cell surface. Consistent with previous reports [3, 26], an analogous distribution was observed when EYFP-tagged synaptotagmin I (SytI-YFP) was co-expressed with CFP-F. In contrast, exogenous synaptophysin I fused to EYFP (SypI-YFP) was targeted to intracellular compartments and no major co-localization with CFP-F was visible (Fig. 1A).

Further evidence for prominent plasma membrane-localization of exogenous VAMP2 was obtained by surface staining of living VAMP2-YFP-expressing cells at 4°C with an antibody to the fluorescent epitope, which is exposed to the external milieu upon targeting of the chimera to the plasma membrane (Fig. 1B). The distribution of anti-FP immunoreactivity largely overlapped with that of intrinsic VAMP2-YFP fluorescence, indicating that the bulk of the exogenous protein was associated with the plasma membrane. Measurement of the immunoreactivity of surface-associated VAMP2-YFP relative to the total intrinsic fluorescence of the chimera in individual transfected cells revealed a dose-dependent linear increase in the fraction of exogenous VAMP2 targeted to the plasma membrane (Fig. 1C).

Consistent with its exclusive binding to the surface-localized chimera, the anti-FP antibody was effectively removed by acid-treatment of cells prior to fixation. Since fluorescence of GFP and its spectral variants is pH-sensitive [27, 28], acid-treatment also led to selective quenching of fluorescence of surface-associated VAMP2-YFP, thus allowing the exclusive detection of the chimera localized to intracellular compartments (Fig. 1B). We exploited this effect to provide a semi-quantitative measurement of the amount of exogenous VAMP2 present at the cell surface, based on quantification of YFP fluorescence intensity in VAMP2-YFP-expressing cells subjected to acid-treatment or left untreated before fixation. Quantification revealed that  $84 \pm 3\%$  of VAMP2-YFP fluorescence derived from surface-targeted chimera (mean  $\pm$  s.d.; n=70-90 cells per condition).

Immunofluorescence analysis showed that intracellular VAMP2-YFP was associated with endosomes positive for both TfR and Rab5, likely reflecting its constitutive recycling route (Fig. 1D). In order to study endocytosis of exogenous VAMP2, VAMP2-YFP-expressing cells surface-labeled with the anti-FP antibody at 4°C, were subsequently incubated at 37°C for 20 minutes and processed for immunodetection of the internalized antibody after surface stripping of the residual antibody. Several endocytic vesicles containing VAMP2-YFP were observed, indicating that internalization of ectopically expressed VAMP2 was not prevented in this heterotypic context (Fig. 1E).

### **Synaptophysin I interacts with VAMP2 and controls its subcellular distribution**

It has been reported that synaptophysin I controls the presynaptic targeting of VAMP2 in hippocampal neurons and this effect requires the formation of hetero-complexes between the two proteins [11]. We tested whether such sorting control could be reconstituted in a heterologous system by co-expression of VAMP2-YFP with ECFP-tagged synaptophysin I (SypI-CFP) in HeLa cells. Remarkably, a dramatic change in the distribution of exogenous VAMP2 was observed upon co-expression with synaptophysin I.

In the presence of SypI-CFP, VAMP2-YFP no longer accumulated at the plasma membrane but was selectively targeted to intracellular compartments where the two chimeras largely colocalized (overlap coefficient  $0.97 \pm 0.03$ , mean  $\pm$  s.d.;  $n=40$  cells) (Fig. 2A). This effect does not appear to be specific for HeLa cells, since it was also observed in transformed fibroblasts and epithelial cells of various origins, as well as in primary cultures of astrocytes derived from the rat cortex (data not shown). In contrast, exogenous synaptotagmin I (SytI-YFP) retained a prominent plasma membrane targeting even when it was co-expressed with SypI-CFP (Fig. 2A). At least a subset of organelles bearing both VAMP2-YFP and SypI-CFP contained also endogenous TfR (Fig. 2B). Synaptophysin I expression approximately doubled the number of VAMP2-YFP-positive puncta but did not vary the overlap between VAMP2-YFP and TfR (Fig. 2C).

The specificity of the effect of synaptophysin I on VAMP2 sorting may rely on the formation of VAMP2-SypI hetero-dimers. The formation of VAMP2-SypI complexes was detected biochemically in cells co-expressing VAMP2-YFP and HA-tagged synaptophysin I by immunoprecipitation with anti-HA antibody. Co-immunoprecipitation also revealed the

formation of hetero-complexes between VAMP2 and a C-terminal truncated synaptophysin I which completely lacks the cytosolic tail of the protein (Fig. 2D).

### **Synaptophysin I selectively directs VAMP2 to intracellular compartments**

To unequivocally determine whether synaptophysin I-mediated relocalization of VAMP2 impinges on its plasma membrane targeting, membrane proteins of transfected cells were biochemically isolated following a surface biotinylation assay. Plasma membrane proteins of cells expressing VAMP2-YFP and either synaptophysin I, C-terminal truncated synaptophysin I or the endosomal component Rab11 were incubated with biotin at 4°C and labeled proteins were recovered by streptavidin-Sepharose precipitation (Fig. 3A). Densitometry revealed that synaptophysin I expression led to a 70% reduction in the steady-state levels of exogenous VAMP2 at the plasma membrane (Fig. 3B). Importantly, the levels of surface-associated TfR were unaffected by synaptophysin I expression (Fig. 3C). Decrease in the surface levels of VAMP2 was not observed upon expression of either C-terminal truncated synaptophysin I or Rab11 (Fig. 3B). We chose Rab11 as a control for the effects of synaptophysin I on sorting since in non-neuronal cells both proteins localize to recycling endosomes and influence cholesterol levels in these compartments when expressed at high doses [29] (data not shown).

We sought to study the effect of synaptophysin I on VAMP2 sorting at the single-cell level. To this purpose, cells co-expressing SypI-CFP and VAMP2-YFP were surface stained with an anti-FP antibody to detect plasma membrane-associated VAMP2-YFP (Fig 4A). The specificity of this staining was ensured by the fact that only surface-targeted VAMP2, but not synaptophysin I, exposes the fluorescent tag to the exterior of the cell. The ratio between surface-associated VAMP2-YFP immunoreactivity to total intrinsic fluorescence of the chimera was measured and correlated to SypI-CFP fluorescence in individual cells (Fig. 4C). This analysis showed that the relative amount of plasma membrane-associated VAMP2-YFP was inversely related to the levels of expression of SypI-CFP.

A similar analysis was carried out in cells co-expressing VAMP2-YFP and either ECFP-tagged C-terminal truncated synaptophysin I (SypI $\Delta$ C-CFP) or ECFP-tagged Rab11 (CFP-Rab11). The subcellular localization of SypI $\Delta$ C-CFP was similar to the distribution of full-length synaptophysin I, with a preferential targeting to intracellular compartments which also contained exogenous VAMP2. Likewise, exogenous VAMP2 was present in CFP-Rab11-positive

endosomes. However, neither SypI $\Delta$ C-CFP nor CFP-Rab11 exhibited the ability to exert a dose-dependent control of VAMP2-YFP localization observed with full-length synaptophysin I (Fig. 4A,C).

To further substantiate the specificity of the effects of synaptophysin I on VAMP2 sorting we examined whether synaptophysin I could perturb the constitutive trafficking of syntaxin 13, a single-pass membrane protein which recycles between the plasma membrane and TfR-positive endosomal compartments [30]. ECFP was fused to the intraluminal carboxy-terminal tail of syntaxin 13 and plasma membrane targeting of the resulting chimera (syntaxin13-CFP) was revealed by surface staining with anti-FP antibody upon co-expression with SypI-YFP (Fig. 4B,C). The ratio between surface-associated immunoreactivity to total intrinsic fluorescence of syntaxin13-CFP did not vary upon SypI-YFP expression (Fig. 4C). Thus, synaptophysin I selectively influences VAMP2 sorting without affecting the distribution of markers of the endosomal recycling pathway.

### **Synaptophysin I does not affect the rate and extent of VAMP2 endocytosis**

The dose-dependent decrease in the surface levels of VAMP2 induced by synaptophysin I might be a consequence of enhanced VAMP2 endocytosis from the plasma membrane. To test this possibility cells co-expressing VAMP2-YFP and either soluble ECFP, used as a control, or SypI-CFP were surface labeled at 4°C with a cleavable biotin derivative (NHS-SS-biotin). Cells were warmed to 37°C for various periods of time to allow endocytosis of surface labeled proteins, and the label remaining at the plasmalemma was removed by reduction of the disulfide linkage prior to streptavidin-Sepharose precipitation (Fig. 5A,B). A significant amount of exogenous VAMP2 was internalized within 15 minutes of incubation at 37°C, becoming resistant to disulfide reduction. Remarkably, synaptophysin I did not influence the internalization rate of either VAMP2 or TfR precipitated from the same extracts.

Precipitation of biotin-labeled VAMP2-YFP and SypI-CFP from the same cell extracts allowed direct comparison of their internalization kinetics. VAMP2 and synaptophysin I showed a fairly similar rate of internalization during the first 15 minutes of chase, although the latter exhibited faster recycling to the plasma membrane in the next 30 minutes (Fig. 5C).

To obtain morphological evidence for the coupling between VAMP2 and synaptophysin I along the endocytic pathway, VAMP2-YFP was surface stained at 4°C with an anti-FP antibody in

cells co-expressing SypI-CFP. Cells were chased at 37°C for 7 minutes to allow endocytosis, and processed for immunodetection of internalized VAMP2-YFP after removal of surface-associated antibody. SypI-CFP was detected in virtually all vesicles bearing endocytosed VAMP2-YFP, suggesting that internalization of the two proteins occurs through a common intermediate (Fig. 5D).

### **Recycling at the plasma membrane is dispensable for synaptophysin I-directed sorting of exogenous VAMP2**

We sought to investigate whether synaptophysin I-mediated control of VAMP2 sorting occurred at the plasma membrane. Thus, we tested whether expression of dynamin bearing the K44A mutation, which inhibits endocytosis in a dominant negative manner [31], impaired the ability of synaptophysin I to recruit VAMP2 to intracellular compartments. Expression of dynamin K44A effectively abolished internalization of fluorescein-conjugated transferrin (Supplementary Figure 1). Cells expressing VAMP2-YFP, SypI-CFP and either wild-type dynamin or the K44A mutant were analyzed 3 days after transfection. VAMP2-YFP was mainly associated with SypI-CFP-positive intracellular compartments both in cells expressing wild-type dynamin and in those expressing the K44A mutant (the overlap coefficient for VAMP2-YFP versus SypI-CFP was  $0.94 \pm 0.05$  and  $0.96 \pm 0.06$  in the presence of wild-type or mutant dynamin, respectively; mean  $\pm$  s.d.; n=20 cells per condition) (Fig. 6A).

The possibility that endocytic blockade could impair the formation of VAMP2/SypI hetero-complexes was assessed biochemically. VAMP2 was co-precipitated with synaptophysin I with equivalent efficiency in cells expressing either wild type or mutant dynamin (Fig. 6B).

As an alternative approach to test the contribution of trafficking at the plasma membrane in the synaptophysin I-regulated sorting of VAMP2, we exploited the dominant negative  $\alpha$ -SNAP L294A mutant, whose expression inhibits fusion at the plasma membrane by preventing SNARE complex disassembly [32]. Cells expressing VAMP2-YFP, SypI-CFP and either wild-type  $\alpha$ -SNAP or the L294A mutant were analyzed 3 days after transfection. The recruitment of VAMP2-YFP to SypI-CFP-positive intracellular organelles was not impaired by  $\alpha$ -SNAP L294A expression (the overlap coefficient for VAMP2-YFP versus SypI-CFP was  $0.97 \pm 0.04$  and  $0.95 \pm 0.04$  in the presence of wild-type or mutant  $\alpha$ -SNAP, respectively; mean  $\pm$  s.d.; n=23 cells per condition) (Fig. 6C). Similar results were obtained when cells were transfected with the

$\alpha$ -SNAP constructs 36 hours prior to transfection of the VAMP2-YFP and SypI-CFP plasmids, in order to allow accumulation of the mutant protein (data not shown).

Collectively, these results indicate that trafficking at the plasma membrane is dispensable for both formation of VAMP2/synaptophysin I complexes and synaptophysin I-directed sorting of exogenous VAMP2.



## DISCUSSION

This study shows that in a heterotypic context which lacks a specific pathway for SV biogenesis, synaptophysin I directs the sorting of VAMP2 to vesicles exhibiting low availability for constitutive exocytosis. In contrast, synaptophysin I does not influence the subcellular distribution of other membrane proteins, such as exogenous synaptotagmin I or markers of the endosomal recycling pathway. The evidence that synaptophysin I retains the ability to control VAMP2 sorting in the absence of a neuronal context emphasizes the intrinsic functional independence of the sorting determinants which direct the trafficking of SV proteins.

In keeping with previous studies reporting a differential subcellular localization for SV proteins heterologously expressed in non-neuronal cells [3, 26, 33-35], we have found that in HeLa cells synaptophysin I localizes to organelles partially overlapping with TfR-positive endosomes, while both synaptotagmin I and VAMP2 are primarily targeted to the plasma membrane.

It has been recently shown that in neurons during recycling SVs retrieve some important components, such as VAMP2 and synaptotagmin I, from large surface reservoirs whose size is determined by the rates of SV exocytosis and endocytosis [36-38]. Whereas the surface localization of synaptotagmin I in non-neuronal cells has been ascribed to the absence of a neuro-specific mechanism required for its internalization [26, 39], in the case of VAMP2 we show that the protein is readily endocytosed and recycled through TfR-/Rab5-positive endosomes. Therefore, the dose-dependent accumulation of VAMP2 at the plasma membrane in non-neuronal cells is likely to result from saturation of the capacity of the endocytotic machinery.

In neurons, VAMP2 accumulates at the plasma membrane when overexpressed in the absence of stoichiometric amounts of synaptophysin I, whereas the proportionate increase in synaptophysin I expression levels restores the exclusive localization of VAMP2 to synapses [11]. However, since the balance between exocytosis and endocytosis of membrane proteins determines their localization to specific organelles or subcellular compartments, it is essential to establish whether synaptophysin I governs the sorting of VAMP2 either by facilitating its targeting to SV precursors along the exocytic pathway or by favoring its recruitment from the plasma membrane. The latter possibility is inconsistent with our endocytosis assays showing that the extent and kinetics of VAMP2 internalization are not influenced by synaptophysin I expression. In addition,

a role for the plasma membrane as a major domain for VAMP2 sorting by synaptophysin I is not supported by the observation that expression of dynamin or  $\alpha$ -SNAP mutants which block trafficking at the cell surface has no effects on the recruitment of VAMP2 to intracellular compartments. Nonetheless, VAMP2 and synaptophysin I are recycled at the cell surface perhaps through a common endocytic intermediate, consistent with previous observations in neurons [27] and PC12 cells [40].

Thus, our results indicate that the essential step in order for synaptophysin I to direct VAMP2 sorting occurs along the exocytic pathway, at either the TGN or endosomal compartments, prior to delivery to the cell surface. The evidence that the plasma membrane is the first membrane acceptor at the level of which VAMP2 functions as a v-SNARE [41] seems to suggest that synaptophysin I and VAMP2 are co-sorted at the level of the TGN into vesicles directed to the cell surface. Vesicles bearing synaptophysin I and VAMP2, probably assembled in hetero-complexes as documented for SVs [16-18, 20], are likely to exhibit a reduced propensity to be consumed by constitutive exocytosis. Indeed, interaction with synaptophysin I limits VAMP2 availability for the formation of SNARE complexes [18] and stimulation-induced release of VAMP2 from synaptophysin I appears to be a prerequisite for SV fusion [20-22]. Consistently, the number of VAMP2-positive vesicles displays a 2-fold increase when synaptophysin I is present. In addition, since this effect is accompanied by a 70% decrease in the surface levels of VAMP2, it appears that synaptophysin I increases either the number of VAMP2 molecules accommodated onto each vesicle or the vesicle size. The latter possibility might be compatible with the documented interaction of synaptophysin I with cholesterol [42], as well as with the hypothesized role of the protein in vesicle biogenesis [42]. Indeed, when expressed at exceedingly high doses, synaptophysin I has the ability to selectively alter the morphology and recycling properties of TfR-positive endosomes, leading to accumulation of cholesterol in these organelles (our unpublished observations).

Protein-protein interaction is not sufficient in order for synaptophysin I to govern the sorting of VAMP2. Indeed, C-terminal truncated synaptophysin I still interacts with VAMP2, but is unable to determine its intracellular sorting. Thus, the long C-terminal cytoplasmic domain is not required for formation of either synaptophysin I homo-oligomers [20] or hetero-complexes with VAMP2, but appears to be important for the control of VAMP2 targeting to intracellular compartments. This domain is phosphorylated by Src and  $\text{Ca}^{2+}$ /calmodulin-dependent protein

kinase II [43-45] and contains binding sites for dynamin I [46] and the AP-1 adaptor protein  $\gamma$ -adaptin [47], as well as an internalization signal required for constitutive endocytosis of synaptophysin I [48]. Hence, the control of VAMP2 sorting by synaptophysin I might require both a direct interaction between the two proteins [23] and additional modulatory processes, such as post-translational modifications or recruitment of other factors to the complex, which are prevented by deletion of the cytoplasmic C-terminal domain.

The view of VAMP2 sorting by its negative regulator, synaptophysin I, at early stages along the secretory pathway has immediate corollaries bearing on the control of the delivery of SV proteins from the neuronal cell body to synapses mediated by VAMP2-positive axonal membrane carriers [49]. This model implicates synaptophysin I in escorting VAMP2 to the sites where exocytosis must take place exclusively after the arrival of the appropriate stimulus. This mechanism would ensure that SV precursors endowed with VAMP2 are kept unable to fuse until they reach their proper destination.

The reduced propensity for vesicle fusion imposed by synaptophysin I does not entirely account for the effect of the protein on VAMP2 sorting. Indeed, the selective control exerted by synaptophysin I on VAMP2 targeting implies that along the exocytic pathway synaptophysin I directs VAMP2 to vesicles distinct from those mediating surface delivery of the other membrane proteins tested, including exogenous synaptotagmin I and markers of the endosomal recycling pathway. These vesicles might be equivalent to the electron-translucent microvesicles containing exogenous synaptophysin I described previously in epithelial cells [50]. These results argue for the coexistence in the same cell of a variety of programs for constitutive exocytosis which are subjected to specific control mechanisms and preferentially exploited by different classes of proteins for their delivery to the cell surface.

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## FIGURE LEGENDS

### **Figure 1. Exogenous VAMP2 is predominantly associated with the plasma membrane.**

(A) Farnesylated ECFP (CFP-F, green) co-expressed with either VAMP2-YFP (left), SytI-YFP (middle) or SypI-YFP (right) (red). (B) (upper panels) Unfixed cells expressing VAMP2-YFP (green in the merge images) surface stained at 4°C with an anti-FP antibody ( $\alpha$ -YFP, red in the merge images). (lower panels) The plasma membrane-associated anti-FP antibody was removed by acid-stripping, which also quenched the intrinsic fluorescence of surface-localized VAMP2-YFP. (C) Surface staining of VAMP2-YFP plotted against the total fluorescence of the chimera. Each dot corresponds to a single cell. ( $R^2=0.82$ ;  $p<0.01$ ) (D) VAMP2-YFP-positive vesicles (green) show partial colocalization (arrowheads) with either TfR or ECFP-tagged Rab5 (red). (E) VAMP2-YFP endocytosis monitored by internalization of surface-associated anti-FP antibody after a 20 minute-incubation at 37°C. A high magnification of the outlined area is shown in the lower panels.

Bar, 7  $\mu\text{m}$  in A; 10  $\mu\text{m}$  in B and the upper panels of E; 2.5  $\mu\text{m}$  in the lower panels of E; 1.6  $\mu\text{m}$  in D.

### **Figure 2. Synaptophysin I interacts with VAMP2 and leads to its redistribution to intracellular compartments.**

(A) Cells co-expressing SypI-CFP (green in the merge images) and either VAMP2-YFP (upper panel) or SytI-YFP (lower panel) (red in the merge images). Synaptophysin I directs VAMP2 to intracellular compartments, whereas it does not influence the plasma membrane localization of synaptotagmin I. (B) A subset of vesicles bearing both VAMP2-YFP and SypI-CFP contain also TfR (arrowheads). (C) Number of VAMP2-YFP-positive intracellular puncta and their colocalization with TfR measured in cells expressing VAMP2-YFP and either soluble ECFP (VAMP2-YFP, light grey bars) or SypI-CFP (VAMP2-YFP/SypI-CFP, dark grey bars) after quenching of surface EYFP fluorescence (mean  $\pm$  s.d.). Synaptophysin I expression increases the amount of VAMP2-positive organelles (Student's t test,  $p < 0.0001$ ,  $n=72$  cells per condition), while it does not affect the extent of overlapping between VAMP2 and TfR ( $n=46$  cells per condition). (D) Cells co-expressing VAMP2-YFP and either ECFP (VAMP2), HA-tagged SypI-

CFP (VAMP2:SypI-HA) or HA-tagged C-terminal truncated SypI $\Delta$ C-CFP (VAMP2:SypI $\Delta$ C-HA) and cells co-expressing SypI-HA and soluble ECFP (SypI-HA) were processed for immunoprecipitation with anti-HA antibody. Western blotting with anti-synaptophysin I (upper panel) and anti-VAMP2 (lower panel) antibodies reveals the presence of hetero-complexes between VAMP2-YFP (arrowhead) and either SypI-HA or SypI $\Delta$ C-HA. Molecular weights are as follows: VAMP2-YFP, 40 kDa; SypI-CFP-HA, 60-65 kDa; SypI $\Delta$ C-CFP-HA, 50-55 kDa.

Bar, 10  $\mu$ m in A; 3.7  $\mu$ m in B.

**Figure 3. Synaptophysin I reduces the accumulation of VAMP2 at the plasma membrane.**

(A) Membrane proteins of cells transfected with constructs expressing VAMP2-YFP and either ECFP, SypI-CFP, SypI $\Delta$ C-CFP or CFP-Rab11 were labeled with biotin at 4°C and isolated by streptavidin-Sepharose precipitation. Arrowheads indicate the position of VAMP2-YFP. (B,C) Quantification of three independent experiments of cell biotinylation (mean  $\pm$  s.d.). For each of the proteins the amount recovered from the streptavidin precipitates was normalized to the amount recovered from the total lysates. The intensity of bands from cells transfected with VAMP2-YFP and ECFP was set to 1. The levels of plasma membrane-associated VAMP2-YFP are reduced upon expression of SypI-CFP but not after the expression of either SypI $\Delta$ C-CFP or CFP-Rab11 (B). In contrast, levels of surface-localized TfR are unchanged in all conditions (C). Molecular weights are as follows: VAMP2-YFP, 40 kDa; SypI-CFP, 60-65 kDa; SypI $\Delta$ C-CFP, 50-55 kDa; CFP-Rab11, 51 kDa; TfR, 85 kDa.

**Figure 4. Synaptophysin I exerts a selective and dose-dependent effect on VAMP2 sorting to intracellular compartments.**

(A) Cells co-expressing VAMP2-YFP (green in the merge images) and either SypI-CFP, SypI $\Delta$ C-CFP or CFP-Rab11 (blue in the merge images) surface stained with an anti-FP antibody to detect plasma membrane-associated VAMP2-YFP (red in the merge images). Despite expression of comparable levels of VAMP2-YFP, the amount of surface-exposed chimera is higher in cells expressing lower levels of wild-type SypI-CFP. (B) Cells co-expressing syntaxin13-CFP (green in the merge image) and SypI-YFP (blue in the merge image), surface stained with an anti-FP antibody to detect plasma membrane-associated syntaxin13-CFP (red in the merge image). (C) Correlation plots show the surface-to-total expression ratios for VAMP2-

YFP (three upper panels) or syntaxin13-CFP (lower panel) plotted against the levels of either SypI-CFP, SypIΔC-CFP or CFP-Rab11. Each dot corresponds to a single cell (200-268 cells per each condition from 3-4 independent experiments).  $R^2$  for exponential fitting is 0.6 for VAMP2-YFP:SypI-CFP ( $p < 0.01$ ) and  $< 0.1$  in the other cases.

Bar, 10  $\mu\text{m}$

**Figure 5. The rate of VAMP2 endocytosis is not influenced by synaptophysin I.**

(A) Endocytosis of biotin-labeled membrane proteins in cells expressing VAMP2-YFP and either soluble ECFP (VAMP2) or SypI-CFP (VAMP2:SypI). After surface labeling with biotin at 4°C, cells were incubated at 37°C for the indicated times to allow endocytosis. Prior to precipitation, surface-associated biotin was removed by cleavage of the disulfide bond. The anti-FP antibody reveals the exogenous proteins. The anti-TfR antibody detects biotin-labeled receptors precipitated from the same cell extracts. Note that surface levels of VAMP2 are reduced by synaptophysin I. (B) Quantification of the data from three independent experiments similar to that shown in A. The data are plotted as the fraction of the amount of protein recovered with respect to cells not treated to remove surface label (surface). In each lane the amount of protein recovered from the precipitates was normalized to the amount recovered from the total lysates. (C) Comparison between the rates of VAMP2-YFP (grey) and SypI-CFP (blue) internalization in cells co-expressing the two chimeras. Points in B and C represent the mean ( $\pm$  s.d.). (D) VAMP2-YFP endocytosis during a 7-minute chase at 37°C in cells co-expressing VAMP2-YFP and SypI-CFP, monitored by internalization of surface-bound anti-FP antibody ( $\alpha$ -YFP). Bottom panels show a magnification of the region outlined in the upper panel. Synaptophysin I is associated with vesicles containing endocytosed VAMP2 (arrowheads).

Bar 10  $\mu\text{m}$  in D, upper panels, and 2.5  $\mu\text{m}$  in D, bottom panels.

**Figure 6. Trafficking at the plasma membrane is dispensable for synaptophysin I-directed sorting of VAMP2.**

(A) Cells triple-transfected with expression plasmids for VAMP2-YFP (red), SypI-CFP (green) and HA-tagged either wild-type or dominant negative (K44A mutant) dynamin in a 1:1:2 ratio. Staining with an anti-HA antibody reveals exogenous dynamin (blue). (B) Cells co-expressing

VAMP2-YFP, SypI-CFP and either wild-type dynamin or the K44A mutant were processed for synaptophysin I immunoprecipitation. Western blotting with an anti-VAMP2 antibody reveals the presence of co-precipitated VAMP2. (C) Cells triple-transfected with expression plasmids for VAMP2-YFP (red), SypI-CFP (green) and either wild-type or dominant negative (L294A mutant)  $\alpha$ -SNAP in a 1:1:2 ratio. The anti- $\alpha$ -SNAP antibody was used at concentration exclusively allowing identification of the overexpressed proteins (blue). Note that colocalization between VAMP2-YFP and SypI-CFP that have exited the Golgi complex is visible also in cells in which  $\alpha$ -SNAP L294A severely affects ER-to-Golgi trafficking (right panel). Insets in A and C show magnifications of the square-selected regions.

Bar, 10  $\mu$ m in A and C; 4  $\mu$ m in the insets in A and C.

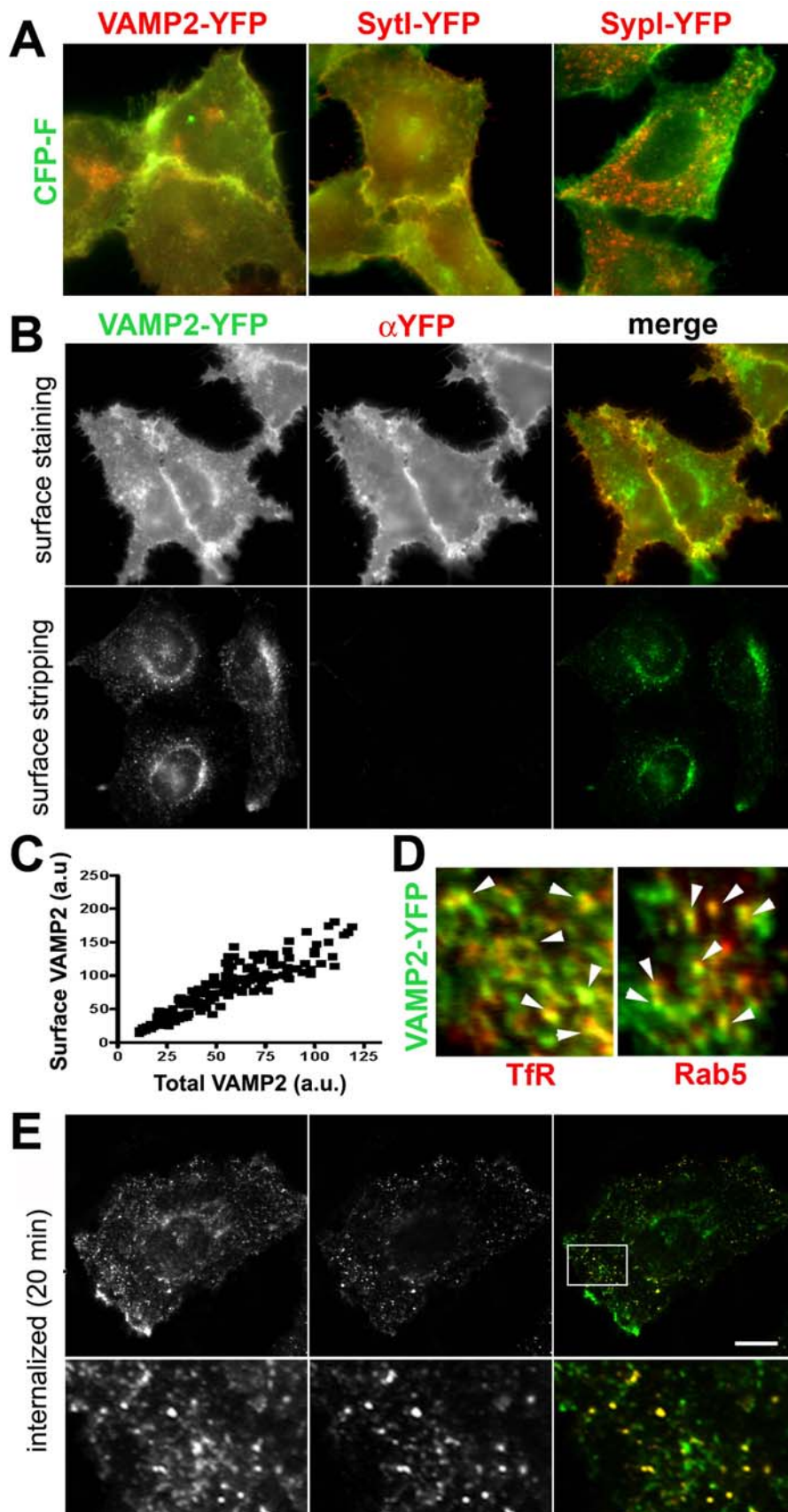


Figure 1

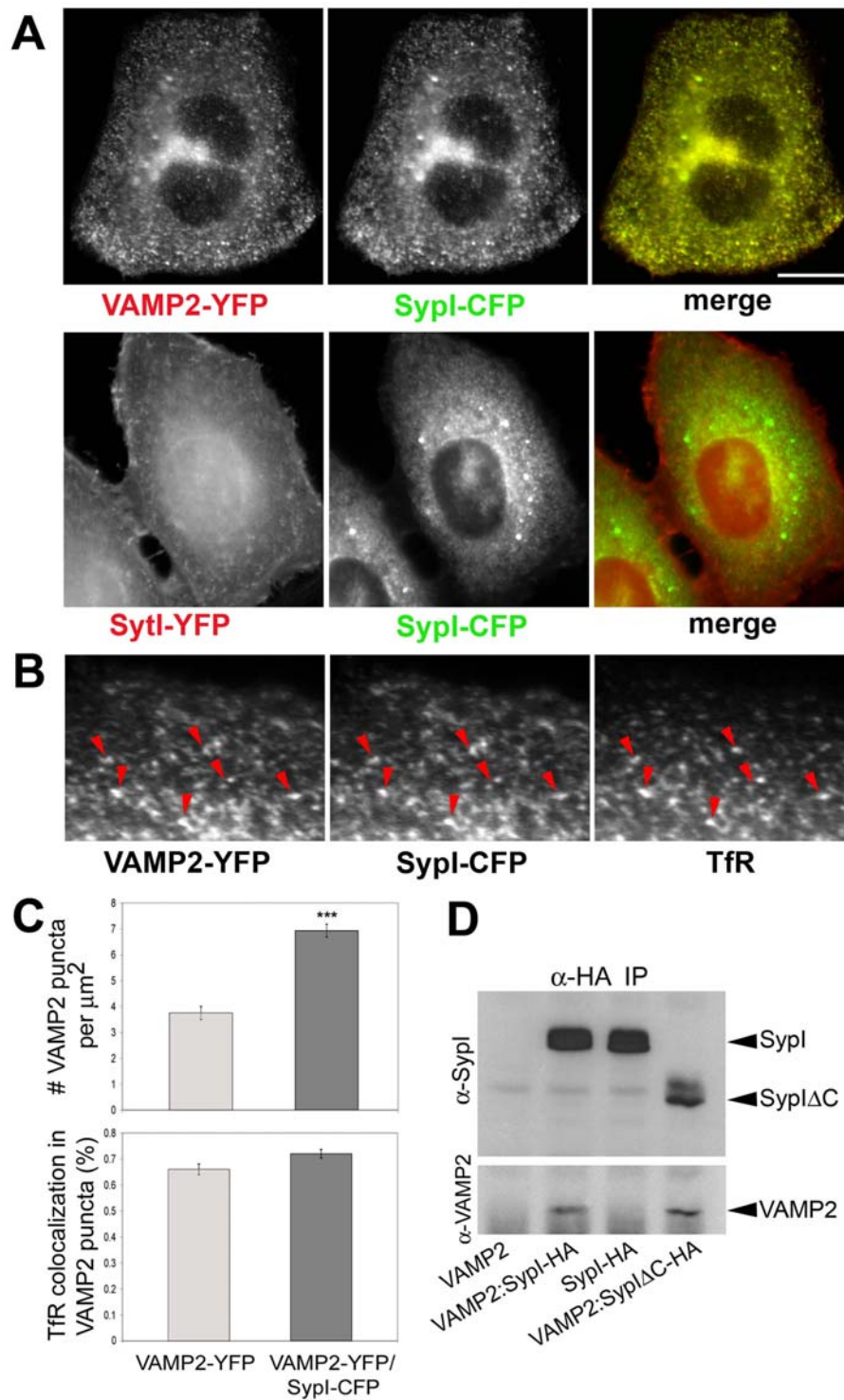


Figure 2



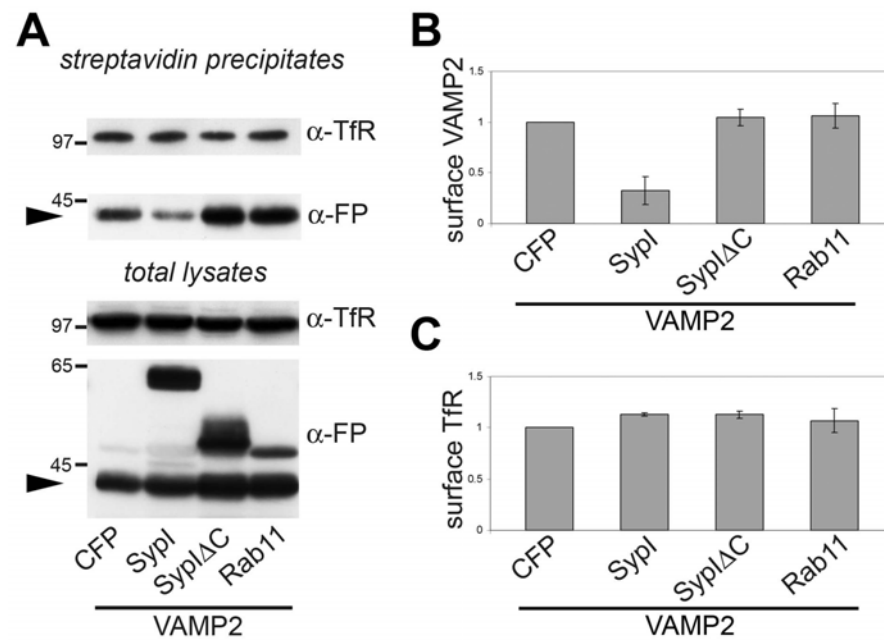


Figure 3

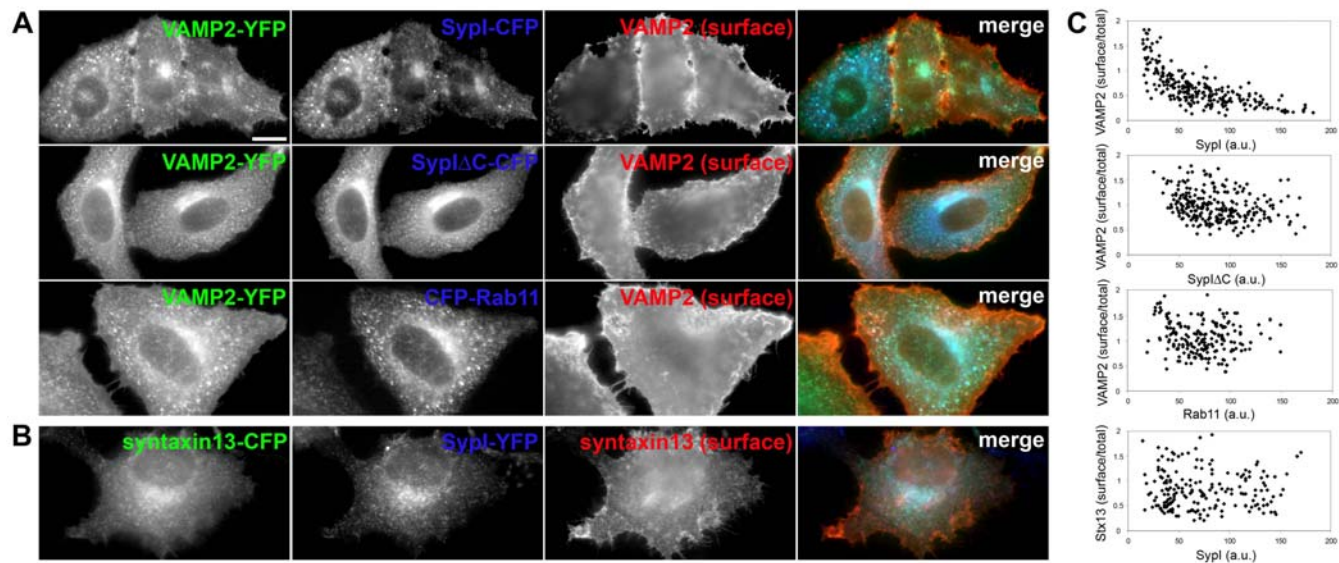


Figure 4

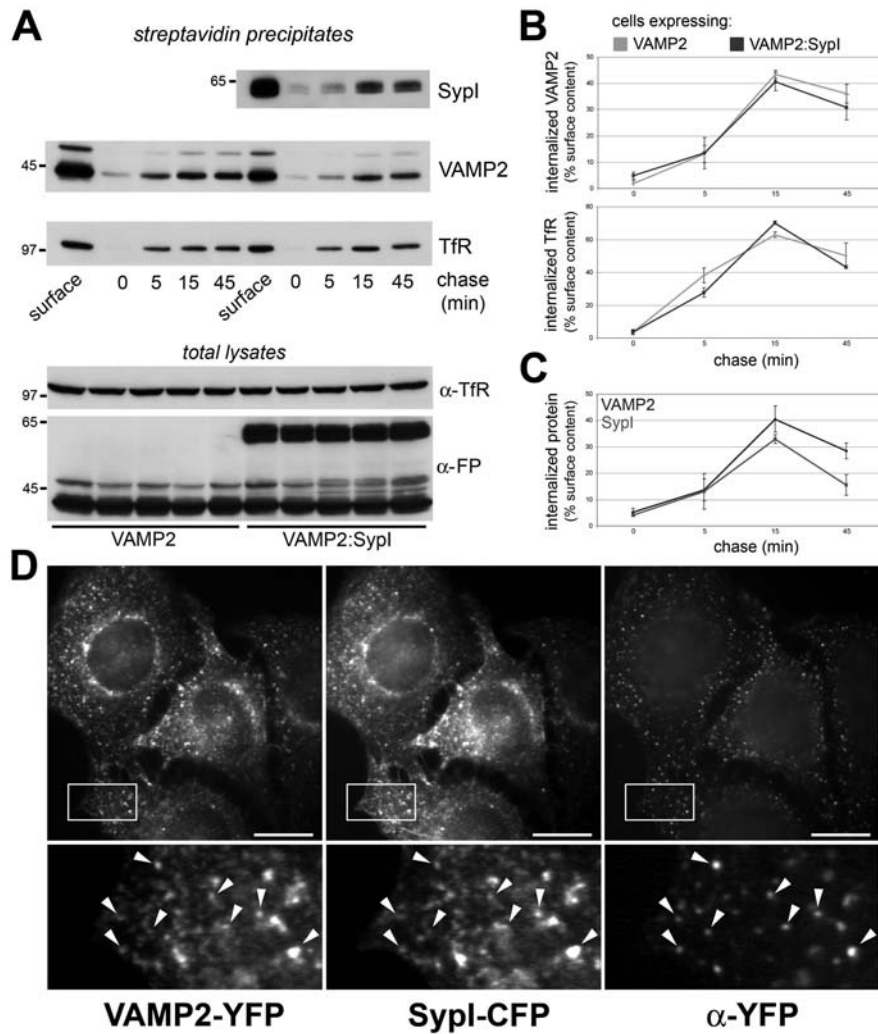


Figure 5

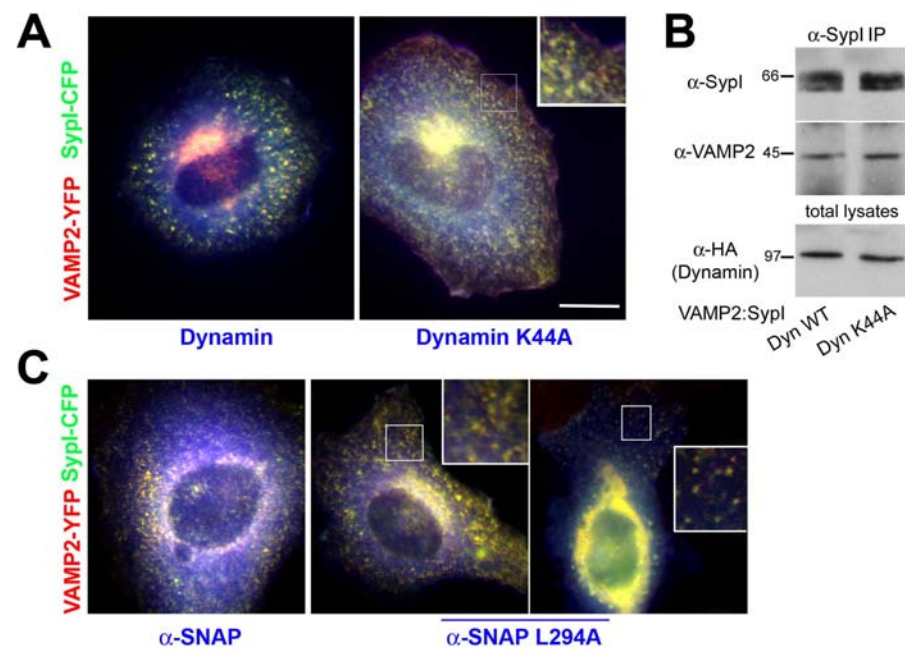


Figure 6