

The Synaptic Vesicle Proteins SV2, Synaptotagmin and Synaptophysin Are Sorted to Separate Cellular Compartments in CHO Fibroblasts

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Abstract. We expressed the synaptic vesicle proteins SV2, synaptotagmin, and synaptophysin in CHO fibroblasts to investigate the targeting information contained by each protein. All three proteins entered different cellular compartments. Synaptotagmin was found on the plasma membrane. Both SV2 and synaptophysin were sorted to small intracellular vesicles, but synaptophysin colocalized with early endosomal markers, while SV2 did not. SV2-containing vesicles did not have the same sedimentation characteristics as

authentic synaptic vesicles, even though transfected SV2 was sorted from endosomal markers. We also created cell lines expressing both SV2 and synaptotagmin, both synaptotagmin and synaptophysin, and lines expressing all three synaptic vesicle proteins. In all cases, the proteins maintained their distinct compartmentalizations, were not found in the same organelle, and did not create synaptic vesicle-like structures. These results have important implications for models of synaptic vesicle biogenesis.

MUCH local neuronal communication occurs via chemical signaling. Small messenger molecules, or classical neurotransmitters, are concentrated in small, clear vesicles that fuse with the plasma membrane and release their contents in response to cellular demand. Once the transmitter is released, vesicles are reformed locally, refilled and can undergo multiple signaling cycles. The mechanisms controlling synaptic vesicle formation and recycling are poorly understood. We do know that mature synaptic vesicles contain a specific, well characterized set of integral membrane proteins (Südhof and Jahn, 1991), but it is not clear how this composition is achieved and maintained.

One clue has come from the analysis of non-neuroendocrine cells transfected with the synaptic vesicle-specific protein synaptophysin (p38). Synaptophysin is sorted to a small, endocytic vesicle population when expressed in most non-neuroendocrine cell lines (Leube et al., 1989; Johnston et al., 1989; Clift-O'Grady et al., 1990; Cameron et al., 1991; Linstedt and Kelly, 1991). This result shows that non-neuronal cells can recognize and sort synaptic vesicle proteins, and further suggests that synaptic vesicles are endocytosed through conventional coated pit recycling with endocytic markers like the transferrin receptor, and are later restricted to a distinct vesicle population. In addition, these findings suggest that synaptic vesicles evolved from a common recycling pathway present in many cells.

Fibroblasts expressing synaptophysin sort the protein to small intracellular vesicles, but those vesicles do not have the same size and sedimentation behavior as authentic synaptic vesicles (Clift-O'Grady et al., 1990; Cameron et al., 1991; Linstedt and Kelly, 1991). A remarkable characteristic of

synaptic vesicles is their extremely uniform 40-nm diameter and round shape. Endosomes, on the other hand, are typically larger and have a tubulo-vesicular morphology. The difference in size and shape allows synaptic vesicles to be separated from endosomes by differential centrifugation. Synaptophysin from transfected fibroblasts is recovered in the endosomal fractions, rather than in a neuronal-type vesicle population. Although synaptophysin may contribute endosomal targeting signals to the synaptic vesicle, expression of synaptophysin does not direct the formation of a small, uniform synaptic vesicle-like organelle. Another synaptic vesicle protein might play this role. Alternatively, formation of neuronal-type vesicles may require the coexpression of other synaptic vesicle proteins, and might be detected in cell lines programmed to make more than one synaptic vesicle protein.

Fibroblastic cell lines expressing combinations of the synaptic vesicle proteins would also help to clarify the significance of multimeric synaptic vesicle protein interactions observed *in vitro*. Solubilization of synaptic vesicle membranes in several different detergents leads to the recovery of large protein complexes that include the synaptic vesicle proteins SV2, synaptotagmin, synaptophysin, and rab3A, as well as the vacuolar proton pump and various unidentified components (Bennett et al., 1992a). It was calculated that the majority of the protein in synaptic vesicles could be contained in as few as three of these complexes. If these complexes exist *in vivo*, they could be extremely important in all aspects of synaptic vesicle function, including biogenesis and recycling.

We have created fibroblastic CHO cell lines that express

the synaptic vesicle proteins SV2, synaptotagmin, and synaptophysin to test the generality of endosomal synaptic vesicle protein sorting in non-neuroendocrine cells, and to discover if either SV2 or synaptotagmin can create a synaptic vesicle-like compartment in a foreign environment. In addition, we generated cell lines that coexpress combinations of three synaptic vesicle proteins, as well as all three together, to determine if formation of synaptic vesicle-like structures requires expression of multiple synaptic vesicle proteins. These lines also allow us to test the abilities of specific synaptic vesicle proteins to form complexes with one another in vivo.

SV2 is a highly glycosylated integral membrane protein (Buckley and Kelly, 1985). Sequence analysis revealed that the protein is highly homologous to a group of 12 transmembrane domain prokaryotic and eukaryotic transporters including the human glucose transporter (Bajjalieh et al., 1992; Feany et al., 1992; Gingrich et al., 1992). It seems likely that SV2 functions as a transporter, although its substrate(s) has not been identified, and additional channel or enzymatic functions have not been excluded. Synaptotagmin (p65; Matthew et al., 1981), in contrast, appears to be intimately involved in membrane fusion events during synaptic vesicle exocytosis. The protein contains a single transmembrane domain and a cytoplasmic region that has two copies of a region similar to the C2 domain of the phospholipid-dependent protein kinase C (Perin et al., 1990). Synaptotagmin binds both acidic phospholipids and calcium (Brose et al., 1992), and is found in a complex with the α -latrotoxin receptor (neurexin; Ushkaryov et al., 1992). α -latrotoxin is a vertebrate neurotoxin that induces massive exocytosis (Petrenko et al., 1991). Synaptophysin is thought to be involved in a different stage of neurotransmitter release. Based on its topological similarity to gap junctions and its ability to form voltage-dependent channels in black lipid membranes (Thomas et al., 1988), synaptophysin may form a mast cell-like fusion pore, perhaps in combination with the synaptic plasma membrane protein physophilin (Thomas and Betz, 1990). Our data indicate that fibroblasts sort each of the three synaptic vesicle proteins independently, perhaps in keeping with their diverse roles. SV2 appears in a small vesicle population distinct from synaptophysin and synaptic vesicles, and synaptotagmin is targeted to the plasma membrane.

Materials and Methods

Primary Antibodies

mAbs to synaptophysin (SY38) and tubulin and polyclonal antibodies to human transferrin were from Boehringer Mannheim (Mannheim, Germany). ER antibodies were from Jennifer Lippincott-Schwartz (National Institutes of Health, Bethesda, MD). Kelley Moreman (University of Georgia, Athens, GA) donated the anti-mannosidase II antiserum. A mAb to clathrin (CHC5.9) was from BIODESIGN International (Kennebunkport, ME). A mAb directed against the cytoplasmic epitope of the human transferrin receptor (H68.4) was the kind gift of Ian Trowbridge (Salk Institute, La Jolla, CA). William Brown (Cornell University, Ithaca, NY) donated the anti-mannose-6-phosphate receptor antibody. The mAb E9a4 antibody to the lysosomal protein lgp95 was a gift of Brigitte Foellmer (Yale University, New Haven, CT). Reinhard Jahn (Yale University) donated the mAb C1 41.1 against synaptotagmin. The synaptotagmin ectopeptide antiserum against the luminal domain of the protein was from Pietro De Camilli (Matteoli et al., 1992; Yale University). A polyclonal antiserum to synaptophysin was

the gift of Dan Cutler (MRC, London, England). The anti-low density lipoprotein (LDL)¹ receptor (4A4) antibody was from the American Type Culture Collection (ATCC; Rockland, MD).

Cell Culture and Transfection

CHO-K1 (ATCC) cells were grown in glutamine-free Glasgow MEM (GMEM) (GIBCO-BRL, Gaithersburg, MD) before transfection. To obtain a cDNA encoding synaptotagmin for transfection, the synaptotagmin coding region was amplified from a rat brain cDNA library by the PCR. The synaptotagmin clone was sequenced and two changes were found from the predicted amino acid sequence of the previously published rat cDNA (Perin et al., 1990): E188 changed to D, and D374 was altered to G. It is unlikely that these substitutions are PCR-induced errors because both are conserved in the human and *Drosophila* sequences (Perin et al., 1991). The synaptotagmin cDNA was subcloned into the expression vector pEE14 (Celltech, Berkshire, UK), CHO-K1 cells transfected using Lipofectin (GIBCO-BRL) and stable transformants were selected in the presence of 25 μ M methionine sulphoximine (MSX). Lines were subcloned until 100% of the cells expressed synaptotagmin by immunocytochemistry using alkaline phosphatase detection. A synaptophysin cDNA obtained from A. Linstedt and R. Kelly (Clift-O'Grady et al., 1990) was also expressed in CHO cells using the pEE14 expression vector (Bebington and Hentschel, 1987; Stephens and Cocket, 1989).

The vector CDM8 was used to express SV2 (Feany et al., 1992). To obtain stable cell lines expressing multiple synaptic vesicle proteins, CHO-K1 cells or cells expressing SV2 were transfected with the appropriate synaptotagmin and/or synaptophysin expression plasmids. Cell lines were selected and subcloned as above. All cell lines were maintained in GMEM; synaptotagmin and synaptophysin cells were supplemented with 25 μ M MSX.

Several independent isolates of each cell line were analyzed to ensure reproducibility. All isolates exhibited similar sorting patterns, and were selected for high expression levels only.

Actin microfilaments were disrupted by a 2-h treatment with 10 μ g/ml dihydrocytochalasin B (Sigma Chemical Co., St. Louis, MO). Nocodazole (Sigma Chemical Co.) treatment was also for 2 h in 10 μ g/ml of the drug.

Immunofluorescence Labeling

For immunofluorescence analysis cells were typically grown overnight on poly-L-lysine-coated glass coverslips, washed in PBS, fixed in 4% formaldehyde, permeabilized in a buffer containing 500 mM NaCl, 20 mM sodium phosphate, pH 7.4, and 0.3% Triton X-100, incubated with primary antibody overnight at 4°C and detected with fluorescein or rhodamine-conjugated secondary antibodies (Cappel Laboratories, Durham, NC) in the same buffer (Cameron et al., 1991). For transferrin uptake experiments, cells were starved for 1 h in serum-free medium, incubated for an additional hour in serum-free medium supplemented with 20 μ g/ml iron saturated human transferrin (Sigma Chemical Co.) at 37°C at 10% CO₂. Cells were then washed quickly in ice-cold PBS, fixed, and processed for immunofluorescence. Actin was visualized using rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR).

Gradient Fractionation and Organelle Immunolocalization

For gradient fractionation cells were harvested in homogenization buffer containing 100 mM NaCl, 10 mM Hepes, pH 7.4, 1 mM EDTA, 0.1 mM MgCl₂. The cells were then homogenized in homogenization buffer with 1 mM PMSF, 10 ng/ml aprotinin, pepstatin, and leupeptin, by 10 passes past a cell cracker (Berni-Tech Engineering, Saratoga, CA) ball bearing with a 12- μ m clearance. Unbroken cells and debris were removed with a 5-min spin at 2,000 g. Postnuclear supernatants were loaded on 10–50% sucrose gradients or 5–25% glycerol gradients with a sucrose pad. Glycerol gradients were spun for 1 h at 48,000 rpm in a SW50.1 rotor at 4°C; sucrose gradients were spun at least 15 h at 48,000 rpm in the same rotor, at the same temperature. For organelle immunolocalization, magnetic IgG-coated beads (Dynal Inc., Great Neck, NY) were incubated in SY38, 4A4 anti-LDL receptor or SV2 antibody overnight. Beads were washed three times in immunoprecipitation buffer containing PBS with 10 mM EGTA and 1% BSA, and resuspended in immunoprecipitation buffer. For all immunoprecipitations, three peak sucrose gradient fractions were pooled (av-

1. *Abbreviations used in this paper:* GMEM, Glasgow MEM; LDL, low density lipoprotein; MSX, methionine sulphoximine.

erage approximate density of 1.14 g/cc), and various amounts added to the magnetic beads. The mixture was rotated for 2 h at 4°C. Immunoprecipitated organelles were washed three times in immunoprecipitation buffer and solubilized in SDS-PAGE sample buffer.

Western Blot Analysis

Gradient fractions were precipitated with acetone, resuspended in sample buffer, run on 4–15% gradient polyacrylamide gels and transferred onto nitrocellulose. Blots were blocked for 1 h in 50 mM Tris, pH 7.4, 100 mM NaCl, .05% Tween-20, 2% nonfat dry milk and 2% normal goat serum. Primary antibodies were diluted in the same buffer and incubated on the blots for an additional hour. Synaptophysin was detected with SY38; synaptotagmin, Cl 41.4; transferrin receptor, H68.4. Bound primary antibodies were detected using ¹²⁵I-labeled goat anti-mouse IgG (New England Nuclear). Signals were quantified using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). The SV2 signal appeared as a high molecular weight smear, and was quantified by selecting the molecular weight range that contained >90% of the total SV2 signal and scanning this same region on each blot. Synaptotagmin was quantified by scanning the 65-kD band.

Samples in the blot shown in Fig. 2 were electrophoresed, blotted, and detected in parallel to allow comparison of expression levels.

Electron Microscopy

Membranes from the peak sucrose gradient fraction were diluted in homogenization buffer and pelleted for 1 h in an airfuge (150,000 g). Pellets were fixed in freshly prepared 4% formaldehyde, washed with PBS, and blocked for 1 h in PBS with 1% BSA. Membranes were incubated at 4°C overnight with SY38 or anti-SV2, washed three times, incubated for 1 h with gold-conjugated goat anti-mouse IgG, washed three additional times, and processed for electron microscopy as follows. Cell pellets were fixed in 2.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, for 30 min. After a brief wash in PBS, the pellets were postfixated in osmium-ferrocyanide (Karnovsky, M. J. 1971. *J. Cell Biol.* 51:146a) for 1 h at room temperature. The cells were then stained en bloc in 1% uranyl acetate in maleate buffer, pH 6.0, for 1 h at room temperature. Subsequently, the pellets were dehydrated in ethanol and embedded in epon-araldite. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a JEOL 100CX electron microscope (JEOL Ltd., Tokyo, Japan). Cells which were grown on glass coverslips were processed as described above for cell pellets, except that fixation consisted of 2% formaldehyde, 1% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4.

Surface Biotinylation

One confluent 10-cm plate each of SV2 or synaptotagmin expressing CHO cells was washed three times in 50 mM sodium borate, 150 mM NaCl, pH 9.0, and biotinylated by two 15-min incubations at 0°C in 0.5 mg/ml NHS-SS-biotin in borate buffer. Cells were washed, removed in Ca²⁺/Mg²⁺ free PBS, pelleted, membranes solubilized in 1% NP-40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris-HCl, pH 7.4, and divided into two aliquots. The first was immunoprecipitated with antibodies to SV2 or synaptotagmin, and used for determination of total synaptic vesicle protein. The second was treated first with streptavidin agarose and the nonbinding fraction was then immunoprecipitated with antibodies to the synaptic vesicle proteins. Immunoprecipitated material and proteins precipitated by streptavidin agarose were solubilized in SDS-PAGE sample buffer, processed for Western blot analysis and quantitated as above. Percentage of cell surface protein was calculated as the fraction of the total synaptic vesicle protein bound to streptavidin agarose. Values plotted are the mean of three determinations plus or minus the standard error.

Results

Immunofluorescent Localization of SV2 and Synaptotagmin

To investigate the targeting information contained in the SV2 and synaptotagmin primary sequences, we expressed both proteins in CHO fibroblasts. Cell lines expressing synap-

tophysin were used as controls. Previous experiments demonstrated that synaptophysin enters an endosomal compartment in fibroblasts (Leube et al., 1989; Johnston et al., 1989; Clift-O'Grady et al., 1990; Cameron et al., 1991; Linstedt and Kelly, 1991). The intracellular localization of the transfected proteins was examined by immunofluorescence. SV2 (Fig. 1 A) accumulated primarily intracellularly and, like synaptophysin (Fig. 1 B) had a punctate perinuclear and cytoplasmic fluorescent staining pattern. In contrast, synaptotagmin was found in the plasma membrane and filopodia (Fig. 1 C; Feany and Buckley, 1993). The differences in the three patterns did not appear to result from gross overexpression of the proteins because each protein was expressed at lower levels in CHO cells than in rat brain (Fig. 2; see Materials and Methods).

To more precisely localize SV2, we compared SV2 fluorescence with a series of proteins known to reside in distinct subcellular compartments. SV2 staining was clearly different from the pattern of a resident ER protein (Fig. 3, A and B). Although some SV2 protein did localize to the Golgi region, most of the punctate cytoplasmic reactivity was distinct (Fig. 3, C and D). SV2 staining was unlike clathrin immunoreactivity (Fig. 3, E and F), which marks coated pits and vesicles. Similarly, SV2 positive structures did not correspond with the mannose-6-phosphate receptor, a marker for late endosomes and the *trans*-Golgi region (Fig. 3, K and L), or lysosomes, as scored by a resident lysosomal glycoprotein (Fig. 3, M and N). The pattern of SV2 fluorescence did seem similar to that of internalized transferrin, a marker of endocytic vesicles (Fig. 3, G and H), but fluorescent puncta did not exactly correspond. In contrast, synaptophysin immunoreactive puncta appeared to coincide precisely with transferrin positive structures (Fig. 3, I and J).

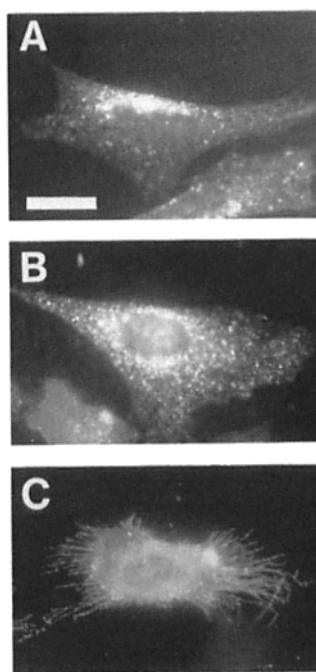


Figure 1. Immunofluorescence microscopy of SV2 (A), synaptophysin (B), and synaptotagmin (C) expressed in fibroblastic CHO cells and detected with a mAb to SV2 (Buckley and Kelly, 1985), SY38, and a mAb to synaptotagmin (Matthew et al., 1981). All subsequent immunofluorescence micrographs are at the same magnification. Bar, 15 μ m.

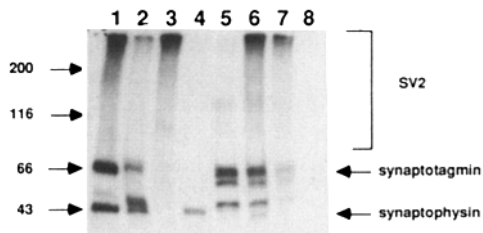


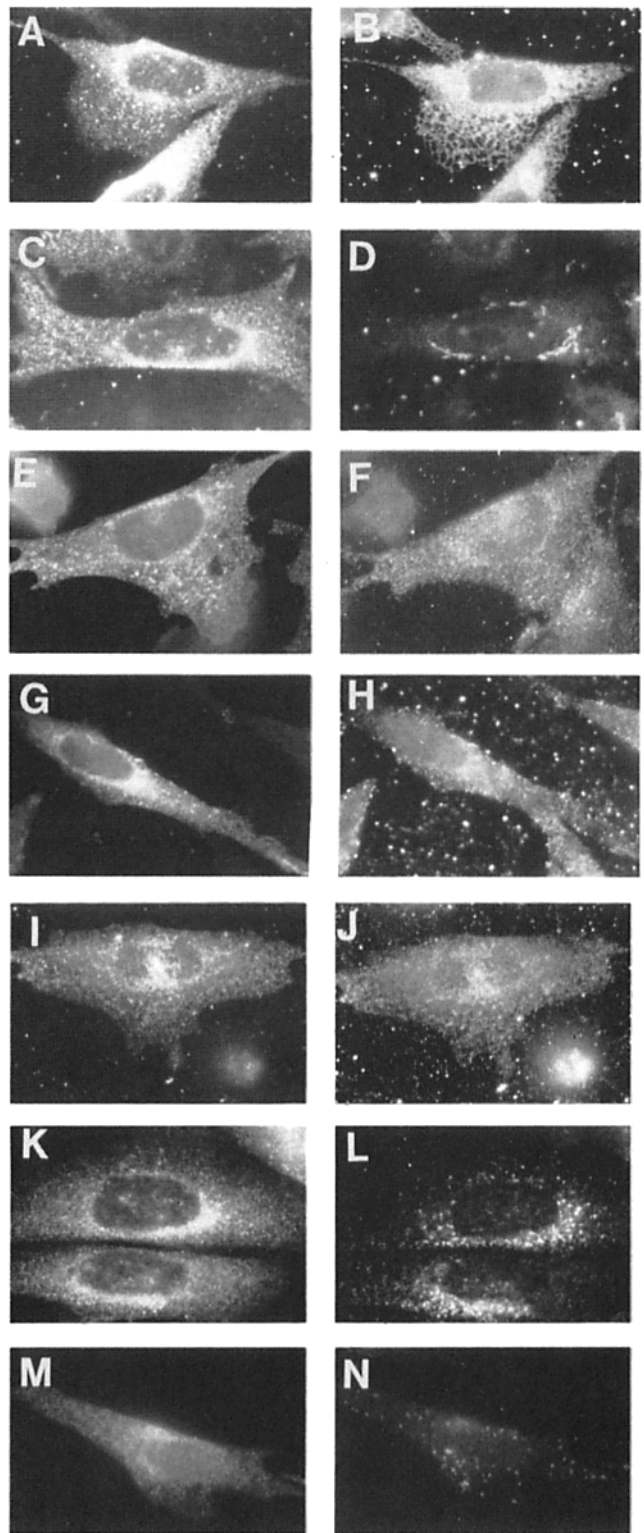
Figure 2. Western blot analysis demonstrates comparable expression of the transgenes. (Lane 1) Immunoblot of total rat brain homogenate incubated with antibodies against SV2, synaptophysin, and synaptotagmin; (lane 2) an equal amount of total PC12 homogenate; (lane 3) SV2 expressing cells; (lane 4) synaptophysin transfected cells; (lane 5) synaptotagmin expressing cells; (lane 6) cells expressing SV2, synaptophysin and synaptotagmin; and (lane 7) cells expressing SV2 and synaptotagmin. Lane 8 shows an immunoblot of cells transfected with the vector alone. Cell lines were fractionated on 10–50% sucrose gradients, fractions immunoblotted and reacted with appropriate SV2, synaptophysin and/or synaptotagmin (C1 41.1) antibodies. An autoradiograph of the peak fraction is shown. Gradient centrifugation was necessary as the levels of transfected protein were too low to be detected reliably in the total cellular homogenate. The transfected cell lines are enriched for the synaptic vesicle proteins approximately fivefold by gradient fractionation. Equal amounts of protein were loaded in each lane. Lanes 5, 6, and 7 have two low molecular weight bands that react with the synaptotagmin antibody. They presumably represent synaptotagmin breakdown products. Arrows indicate molecular masses in kD, and the positions of the proteins SV2, synaptotagmin and synaptophysin.

Gradient Fractionation of SV2 and Synaptotagmin Expressing Cells

To more carefully define the compartments containing the transfected proteins, and to monitor the possible genesis of a synaptic vesicle-like organelle, we performed gradient fractionation of the transfected cell lines. When CHO cells expressing synaptophysin were fractionated on a shallow 10–50% linear sucrose gradient a single sharp peak of synaptophysin immunoreactivity was detected by Western blot analysis (Fig. 4 A). This peak corresponded exactly to the peak of transferrin immunoreactivity, suggesting that the two proteins occupied the same intracellular compartment. In contrast, SV2 immunoreactivity appeared as a much broader peak (Fig. 4 B). Much of the protein fractionated at the same density as the transferrin receptor; however, some SV2 was found in organelles with a higher density. Synaptotagmin also fractionated as a broad peak on sucrose gradients (Fig. 4 C). Again, a portion of the protein was in fractions that had the same density as the transferrin receptor containing endosomes, but a substantial percentage was not.

The rat pheochromocytoma cell line (PC12) has been a useful source of synaptic vesicle-like structures for biochemical characterization. The synaptophysin protein is found in two distinct compartments in this cell line. One cofraction-

Figure 3. Double-label immunofluorescence microscopy of SV2 in comparison with markers of other membrane compartments. (A and B) Double-label immunostaining of SV2 and an antiserum specific for the ER, respectively. (C and D) Double-label im-



munofluorescence for SV2 and mannosidase II, a Golgi protein, respectively. (E and F) Comparison of SV2 with clathrin, respectively. (G and H) Double-label immunostaining of SV2 and internalized human transferrin, respectively. (I and J) Double-label immunofluorescence for synaptophysin and internalized human transferrin, respectively. (K and L) Comparison of SV2 and the mannose-6-phosphate receptor, respectively. (M and N) Double-label immunostaining of SV2 and an antibody specific for the lysosomal glycoprotein lgp95, respectively.

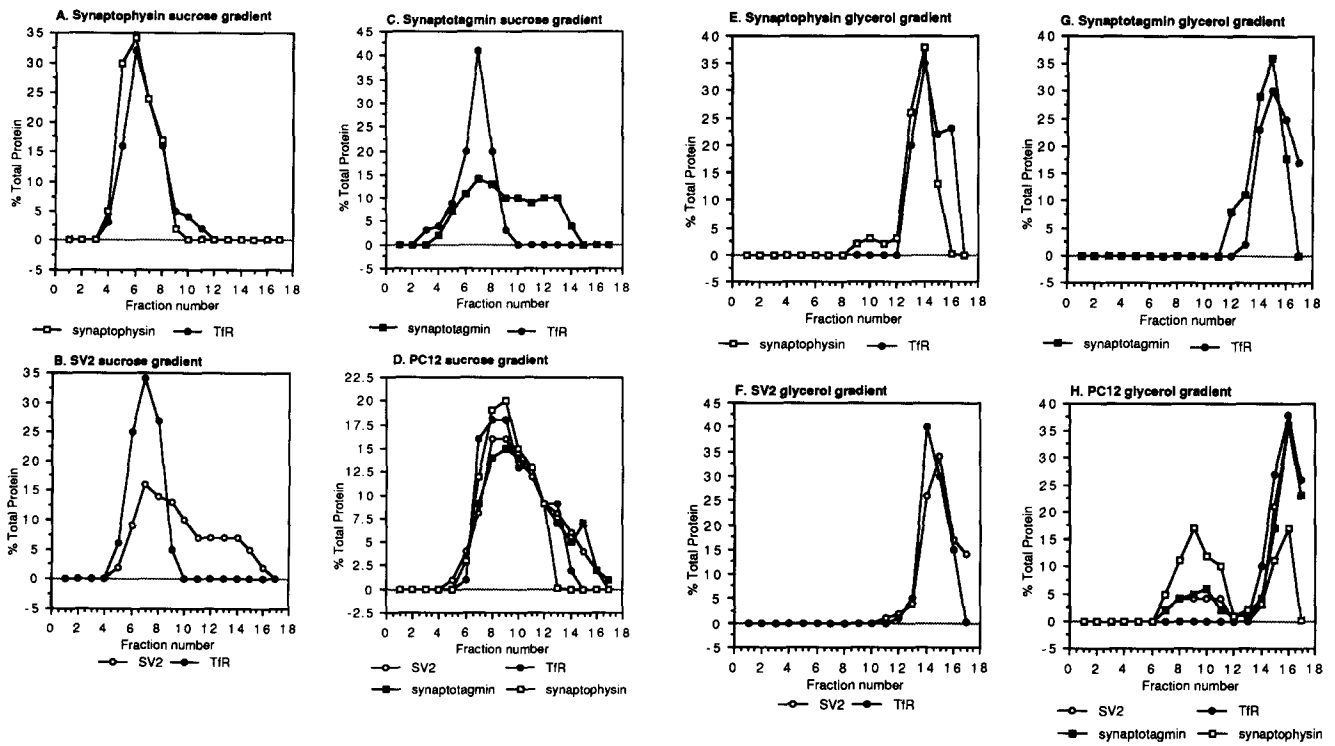


Figure 4. Comparison of synaptophysin, SV2 and synaptotagmin expressing cell lines, and PC12 cells by density and velocity centrifugation. (A-D) Membranes were separated on a 10–50% linear sucrose gradient and fractions immunoblotted for the synaptic vesicle proteins and for endogenous transferrin receptor. (E-H) Membranes were separated on a linear 5–25% glycerol gradient over a 0.5 ml 50% sucrose pad, fractions collected and immunoblotted for the synaptic vesicle proteins and for the transferrin receptor. Fractions are numbered from the top.

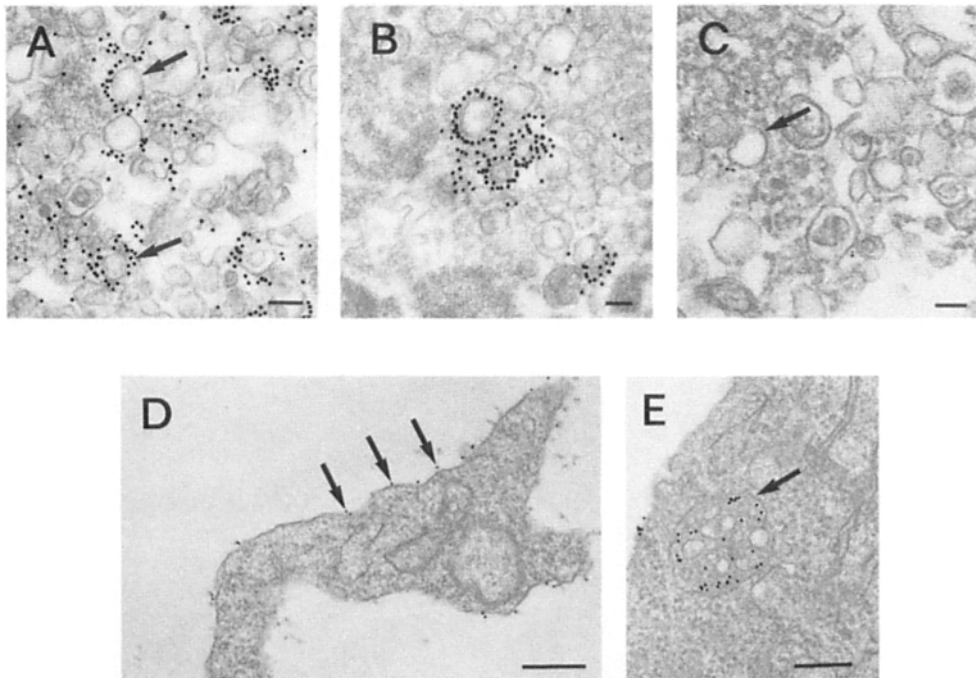


Figure 5. Immunogold labeling of SV2 and synaptophysin containing membranes from peak sucrose gradient fractions, and of synaptotagmin expressing cells. (A) Membranes were recovered from SV2 sucrose gradient fractions, labeled with SV2 antibody and detected with a gold-conjugated secondary antibody. Labeled membranes consist of a relatively uniform vesicle population with an 80-nm diameter. (B) Synaptophysin membranes were labeled with the immunogold technique and reveal similar vesicles. (C) No specific membrane labeling was seen when cells transfected with vector alone were incubated with the SV2 antibody and gold-conjugated secondary antibody. (D) Plasma membrane labeling is apparent when live synaptotagmin expressing cells were

incubated at 37°C with an antibody that recognizes the extracellular domain of synaptotagmin (Matteoli et al., 1992). Specific labeling was detected with gold-conjugated secondary antibody. (E) Occasional multivesicular bodies were labeled after the 37°C incubation. Bars: (A–C) 100 nm; (D) 0.5 μ m; (E) 0.2 μ m.

ates with and contains endosomal markers, the other migrates with authentic synaptic vesicles and contains other synaptic vesicle proteins (Clift-O'Grady et al., 1990; Cameron et al., 1991; Linstedt and Kelly, 1991). By sucrose density gradient analysis both populations of vesicles migrated as one rather broad peak (Fig. 4 D). However, the different sedimentation coefficients allow the two vesicle populations to be resolved on a glycerol velocity gradient (Fig. 4 H). The synaptic vesicle-like population was seen in the upper portions of the gradients and contains SV2, synaptotagmin and synaptophysin, but not transferrin receptor. The synaptic vesicle-like fractions were relatively enriched for synaptophysin as compared with the other two synaptic vesicle proteins.

We confirmed previous reports that synaptophysin is not sorted to a synaptic vesicle-like compartment in CHO cells by separating synaptophysin homogenate on a glycerol gradient (Fig. 4 E). SV2 and synaptotagmin homogenates were also fractionated on glycerol gradients to determine if SV2 and/or synaptotagmin were contained in a vesicle population with the same sedimentation behavior as synaptic vesicles. All of the SV2 and synaptotagmin immunoreactivities were recovered in the heavier fractions that also contain transferrin receptor (Fig. 4, F and G). None of the three synaptic vesicle proteins directed the formation of an organelle with the same sedimentation characteristics as authentic synaptic vesicles.

Immunoelectron Microscopic Localization of SV2 and Synaptotagmin

To further characterize the SV2 containing organelles we performed immunoelectron microscopy on membranes found in the peak fraction of SV2 sucrose gradients. Gold-labeled membranes consisted of vesicles with average diameters of ~80 nm (Fig. 5 A). These vesicles were the same size and shape as synaptophysin containing vesicles fractionated and labeled in a similar manner (Fig. 5 B). No consistent labeling of membranes was seen when cells transfected with vector alone were incubated with the SV2 antibody and gold-conjugated secondary antibody (Fig. 5 C), or when SV2 containing membranes were incubated with the secondary antibody only. We did not observe significant labeling of SV2 containing membranes from the heavier sucrose gradient fractions. The concentration of SV2 in those membranes may have been below our detection limit.

Some synaptotagmin cofractionated with early endosomes as defined by transferrin receptor immunoreactivity (Fig. 4 C). To determine if this synaptotagmin was actually in an intracellular, endosomal compartment not apparent on visual inspection of immunofluorescently labeled cells, we used antibodies that recognize the luminal, or extracellular domain of synaptotagmin (Matteoli et al., 1992). This antiserum has been used to monitor multiple rounds of exo- and endocytosis in neuronal cells. To identify endocytosed synaptotagmin cells were incubated with the primary antibody at 37°C for 1 h, followed by a subsequent incubation with the gold-conjugated secondary antibody at 37°C. This procedure should label synaptotagmin that is endocytosed from the plasma membrane during the second 37°C incubation, as well as any continuously recycling protein. In contrast to the results obtained with neurons, we failed to find a significant amount of labeled synaptotagmin inside the

cell. A few small vesicular profiles and multivesicular bodies were labeled (Fig. 5 E), but the majority of the gold particles were confined to the plasma membrane (Fig. 5 D).

Organelle Immunoprecipitation Confirms that SV2, Synaptotagmin, and Synaptophysin Are Not in the Same Compartment

To confirm that the SV2 protein occupies a vesicular compartment distinct from early endosomes, we used an antibody that recognizes the cytoplasmic tail of the LDL receptor to immunoprecipitate endosomal organelles. While LDL receptor antibody-coated beads removed all of the synaptophysin from peak sucrose gradient fractions, they did not precipitate any of the SV2 (Fig. 6 A). Similarly, although LDL receptor antibody removed an average of 90% of the LDL receptor protein, it did not precipitate synaptotagmin from sucrose gradient fractions containing synaptotagmin and transferrin receptor. Immunoprecipitation of plasma membrane LDL receptor should also precipitate synaptotagmin, but we did not observe significant coimmunoprecipitation. It may be that the transferrin receptor containing gradient fractions used were not highly enriched in plasma membrane. Control beads coated with nonspecific mouse immunoglobulin did not precipitate any of the proteins (Fig. 6 A). To further compare the synaptophysin and SV2 containing organelles, magnetic beads were coated with anti-

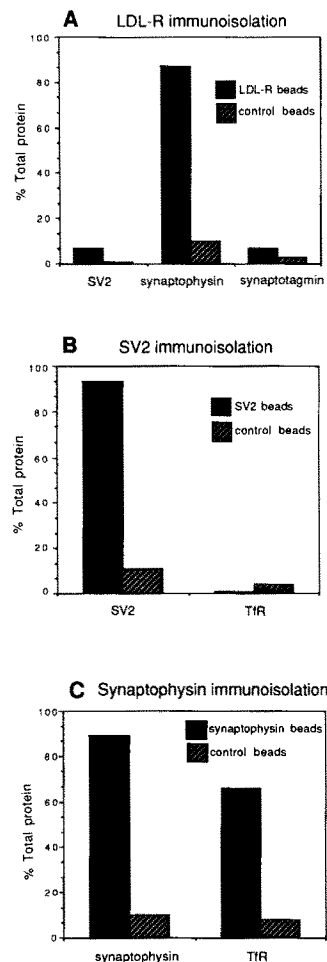


Figure 6. Organelle immunoprecipitation demonstrated that SV2 and synaptotagmin fail to colocalize with the LDL receptor or the transferrin receptor. (A) Organelles from peak sucrose gradient fractions of synaptophysin, SV2 and synaptotagmin expressing CHO cells were immunoprecipitated with magnetic beads coated with antibodies against the LDL receptor. The beads immunoprecipitated the majority of the synaptophysin protein, but very little SV2 or synaptotagmin. (B) SV2 coated beads recovered the SV2 protein, but not the transferrin receptor. (C) Synaptophysin beads immunoprecipitated both synaptophysin and the transferrin receptor. Gradient fractions were treated with beads coated with specific antibody (■) and with beads coated with nonspecific immunoglobulin (▨). The percentage of each specific protein recovered from the washed beads is plotted as a fraction of the total synaptic vesicle or transferrin receptor protein present in the starting homogenate.

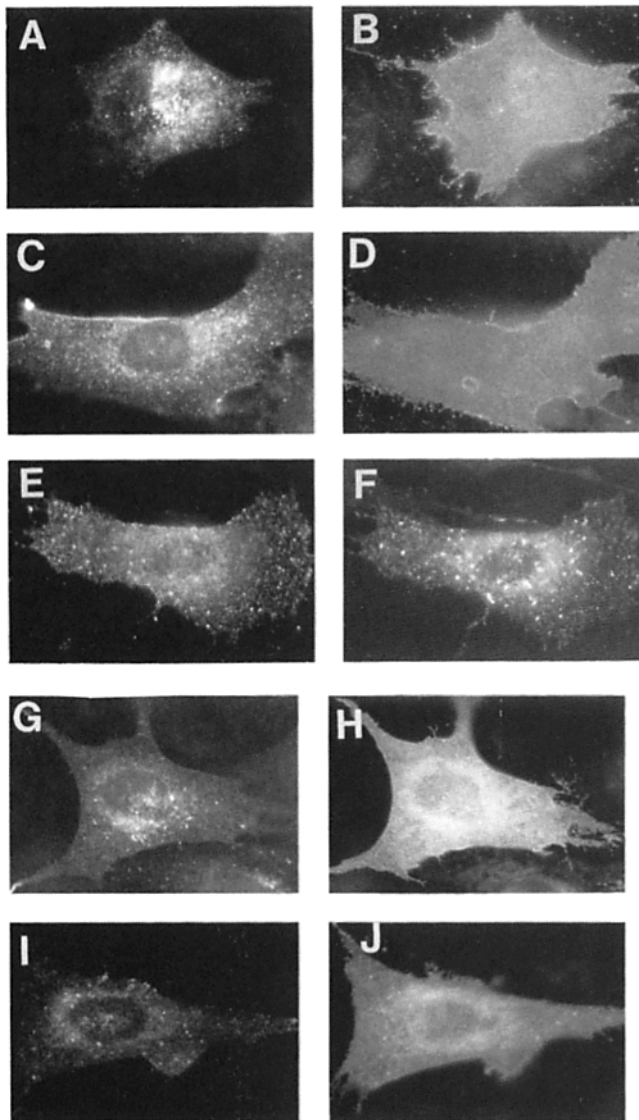


Figure 7. Double-label immunofluorescence microscopy of SV2, synaptotagmin and synaptophysin in cell lines expressing more than one synaptic vesicle protein. (A and B) Double-label immunostaining of SV2 and synaptotagmin, respectively, in a cell line expressing both proteins. (C and D) Double-label immunofluorescence for synaptophysin and synaptotagmin, respectively, in a cell line expressing both proteins. (E and F) Comparison of SV2 and synaptophysin staining, respectively, in a cell line that expresses SV2, synaptotagmin and synaptophysin. (G and H) Double-label immunostaining of SV2 and synaptotagmin, respectively, in a cell line that expresses SV2, synaptotagmin and synaptophysin. (I and J) Double-label immunofluorescence for synaptophysin and synaptotagmin, respectively, in a cell line that expresses SV2, synaptotagmin and synaptophysin. Synaptotagmin was detected with either the mAb 48 (Matthew et al., 1981) or a polyclonal antibody to the extracellular domain of the protein (Matteoli et al., 1992).

bodies against synaptophysin or SV2, and these beads were used to immunoprecipitate synaptophysin and SV2 membranes (Fig. 6, B and C). Most of the synaptic vesicle proteins were precipitated by the appropriate beads. In addition, synaptophysin beads removed most of the transferrin receptor, while SV2 beads did not.

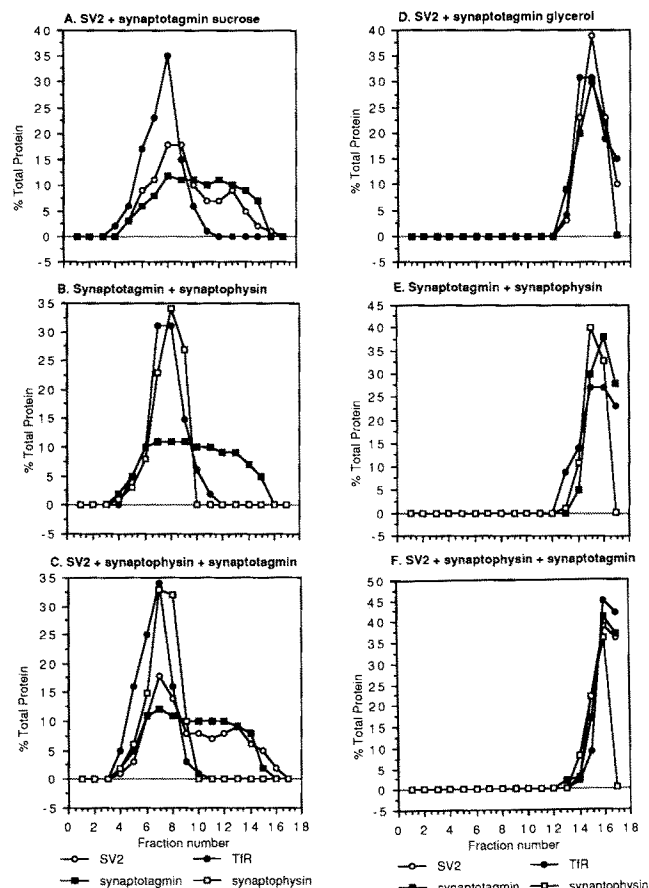


Figure 8. Comparison of cell lines transfected with both SV2 and synaptotagmin (A and D), cells transfected with both synaptophysin and synaptotagmin (B and E), and a line expressing SV2, synaptotagmin and synaptophysin (C and F) by density and velocity centrifugation. (A, B, and C) Membranes were separated on a 10–50% linear sucrose gradient and fractions immunoblotted for the synaptic vesicle proteins and for endogenous transferrin receptor. (D, E, and F) Membranes were separated on a linear 5–25% glycerol gradient over a 0.5 ml 50% sucrose pad, fractions collected and immunoblotted for the synaptic vesicle proteins and for the transferrin receptor. Fractions are numbered from the top.

Analysis of Cell Lines Expressing Multiple Synaptic Vesicle Proteins

When SV2, synaptotagmin, and synaptophysin are expressed singly in fibroblast cell lines they inhabit different cellular compartments. If interactions between the proteins are important in segregating the proteins to the same vesicle, coexpression of one synaptic vesicle protein might alter the distribution of another. We created cell lines that express both SV2 and synaptotagmin, both SV2 and synaptophysin, both synaptotagmin and synaptophysin, and all three proteins together. Immunofluorescent localization revealed that neither SV2 nor synaptophysin coexpression altered the plasma membrane localization of synaptotagmin (Fig. 7, A–D). SV2 and synaptophysin also retained their characteristic punctate cytoplasmic fluorescence patterns. Similarly, in cell lines expressing all three proteins, synaptotagmin was localized to the plasma membrane while SV2 and synaptophysin were retained in an intracellular compartment. The lines that expressed both SV2 and synaptophysin allowed a

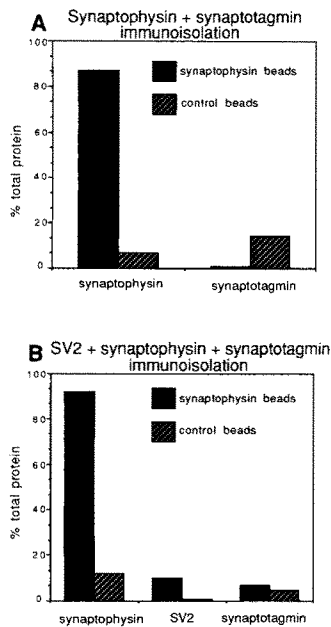


Figure 9. Organelle immunoprecipitation demonstrated that SV2, synaptophysin and synaptotagmin fail to colocalize in cell lines expressing multiple synaptic vesicle proteins. (A) Organelles from peak sucrose gradient fractions of a cell line expressing both synaptophysin and synaptotagmin were immunoprecipitated with magnetic beads coated with antibodies against the synaptophysin. The beads immunoprecipitated the majority of the synaptophysin protein, but very little synaptotagmin. (B) Synaptophysin coated beads recovered synaptophysin, but only a small amount of SV2 or synaptotagmin from a cell line expressing SV2, synaptotagmin and synaptophysin. Gradient fractions were treated with beads

coated with specific antibody (■) and with beads coated with non-specific immunoglobulin (▨). The percentage of each specific protein recovered from the washed beads is plotted as a fraction of the total synaptic vesicle present in the starting gradient fractions.

direct comparison of the immunofluorescence localization of the two proteins. Although the overall patterns of the two proteins were similar (Fig. 3, G and I), labeling of both in the same cell revealed some overlap but also distinct sets of small puncta (Fig. 7, E and F).

Immunofluorescence might not be sensitive enough to identify a small amount of protein that was redirected from one compartment to another in the coexpressing lines. Gradient fractionation and organelle immunoprecipitation were therefore used to more carefully characterize the subcellular compartments occupied by the synaptic vesicle proteins. All three proteins were recovered at densities corresponding to their mobilities in singly transfected cell lines after sucrose gradient fractionation (Fig. 8, A-C). Homogenates from multiply transfected cell lines were then fractionated on glycerol gradients. In each case the synaptic vesicle proteins cofractionated with the transferrin receptor. Even cells expressing SV2, synaptotagmin and synaptophysin failed to sort any of the proteins to a compartment with the same sedimentation behavior as authentic synaptic vesicles (Fig. 8 F). We also immunoprecipitated synaptophysin containing organelles from peak sucrose gradient fractions, and found that in a cell line expressing both synaptophysin and synaptotagmin, and in cells expressing all three proteins, the immunoprecipitated organelles contained very little synaptotagmin or SV2 (Fig. 9). We cannot exclude the possibility of a small degree of overlap between the organelles containing the three proteins.

Surface Biotinylation Demonstrates that the Majority of the Synaptotagmin Protein Is Found on the Plasma Membrane

Analysis thus far suggested that most synaptotagmin was sorted to the plasma membrane, while SV2 and synaptophy-

sin were located primarily intracellularly. Surface biotinylation of plasma membrane proteins was performed followed by determination of the percentage of total SV2 or synaptotagmin labeled (see Materials and Methods). ~90% of the synaptotagmin could be surface labeled with biotin, while very little biotinylated SV2 was recovered (data not shown).

Discussion

SV2 Is Sorted to Small Nonendosomal Vesicles in Fibroblasts

We expressed the synaptic vesicle protein SV2 in CHO fibroblasts and compared its distribution to those of a variety of intracellular proteins by immunofluorescence. None of the markers overlapped precisely with SV2 (Fig. 3, A-N). Internalized transferrin, an early endosomal marker, had the most similar pattern. Several additional methods were used to characterize the SV2 compartment. Sucrose gradient fractionation revealed that the majority of SV2-containing organelles have a density similar to endosomes, as identified by transferrin receptor immunoreactivity (Fig. 4 B). When SV2 containing membranes from sucrose gradients were labeled with gold-conjugated antibodies and examined by EM, SV2 was localized to a population of small, ~80-nm vesicles (Fig. 5 A). These vesicles appeared similar to organelles that contain synaptophysin in CHO cells (Fig. 5 B). However, organelle immunoprecipitation showed that the vesicles that contain SV2 do not contain either the transferrin receptor, or the LDL receptor, a second marker of the endosomal compartment (Fig. 6, A and C). In addition, both immunofluorescence (Fig. 7, E and F) and organelle immunoprecipitation (Fig. 9 B) demonstrated that the majority of the two proteins occupied distinct subcellular compartments in a cell line expressing both SV2 and synaptophysin.

What is the nature of the SV2 compartment? Although we compared SV2 fluorescence with a wide variety of intracellular markers, complete coincidence staining was never observed. The protein does enter a small, relatively light vesicular compartment. In most nonneuroendocrine cells transfected synaptophysin appears in endosomes, but in some cell lines the protein may create a special vesicle that excludes markers of receptor-mediated endocytosis, and that contains primarily synaptophysin (Leube et al., 1989). This result would imply that synaptophysin can direct the organization of a special synaptic vesicle-sized organelle. If any of the synaptic vesicle proteins can actually direct the formation of a novel vesicle in non-neuroendocrine cells, our data suggest that SV2 is more likely than synaptophysin to create such an organelle. SV2 occupies a vesicle that excludes the transferrin and LDL receptors and that has a similar size to synaptic vesicles. However, even if SV2 does segregate to a new organelle, those vesicles do not possess exactly the same biophysical properties as authentic synaptic vesicles. Velocity centrifugation showed that the SV2 vesicles do not have the same sedimentation coefficient as neuronal-type synaptic vesicles (Fig. 4, F and H). Additional synaptic vesicle proteins may be required to alter the sedimentation coefficient. The other membrane components, if any, of the SV2 organelles will help define these vesicles. Examination of the lifecycle of the vesicles to determine if they are endocytic, and if SV2 passes through a defined endosomal compartment, will also be informative.

Synaptotagmin Appears in the Plasma Membrane of Nonneuroendocrine Cells

The presence of synaptotagmin in the plasma membrane of CHO cells was unexpected because all synaptic vesicle proteins normally occupy small intracellular vesicles, as do SV2 and synaptophysin in nonneuroendocrine cells. The plasma membrane localization of synaptotagmin does not appear to be due to overexpression of synaptotagmin protein because all three proteins were expressed at lower levels than in neuronal tissue (Fig. 2 and Results). Significant amounts of synaptotagmin do not accumulate first intracellularly before excess protein enters the plasma membrane. The first detectable synaptotagmin fluorescence occurs in the plasma membrane of cells expressing low levels of synaptotagmin. The fluorescence data does not exclude a minor intracellular pool of synaptotagmin, but this hypothetical compartment is unlikely to be endocytic. Only a small proportion of antibodies to synaptotagmin applied to the outside of the cell are internalized under conditions that allow endocytosis, as assayed by immunoelectron microscopy (Fig. 5, *D* and *E*). The antibody itself is unlikely to interfere with endocytosis because the same antibody was used to follow multiple exo- and endocytotic cycles in neurons, where it had no detectable effect on endocytosis and recycling (Matteoli et al., 1992). Significant amounts of synaptotagmin in an endocytic compartment are also inconsistent with the failure of synaptotagmin to coimmunoprecipitate with the LDL receptor, a marker of endocytic vesicles (Fig. 6 *A*).

Surface biotinylation was performed to directly quantitate the proportion of cell surface synaptotagmin. These studies demonstrated that ~90% of the protein is present on the plasma membrane. In contrast, cell surface SV2 could not be identified. The biotinylation result clearly demonstrates that the majority of transfected synaptotagmin accumulates on the plasma membrane, consistent with the immunofluorescence and immune-electronmicroscopic data.

Expression in a foreign system may be emphasizing an important targeting difference between neuronal synaptotagmin and other synaptic vesicle proteins. Most light immunohistochemical studies have failed to find notable synaptotagmin in neuronal plasma membrane (Matteoli et al., 1992). However, EM immunocytochemistry (Matthew et al., 1981) does show some plasma membrane labeling, and gradient fractionation reveals a significant amount of synaptotagmin (but not synaptophysin) in purified adrenal chromaffin cell plasma membranes (Fournier and Trifaró, 1988). Alternatively, synaptotagmin might remain in the plasma membrane because of an interaction with a plasma membrane protein. Interactions have been described between synaptotagmin and several nerve terminal plasma membrane proteins, including syntaxin, neuexin, and calcium channels (Bennett et al., 1992*b*; Petrenko et al., 1991; Ushkaryov et al., 1992; Hata et al., 1993). Synaptotagmin may be interacting with a nonneuronal homologue of one of these proteins, but the interaction may persist in CHO cells, whereas in the nerve terminal it might normally be transient.

Implications for Synaptic Vesicle Biogenesis and Recycling

Quantal transmitter release is accomplished by fusion of the synaptic vesicle with the presynaptic plasma membrane and release of vesicular contents. New synaptic vesicles are

reformed and refilled at the nerve terminal, but the exact details of recycling remain unclear. Two hypotheses have been advanced to account for the reconstitution of the specialized membrane composition of synaptic vesicles. One model postulates that vesicular membrane components remain segregated during the opening of a transient, mast cell-type fusion pore (Breckenridge and Almers, 1987). Alternatively, synaptic vesicle proteins could mix with plasma membrane constituents, be retrieved in conventional coated pits, and later sorted to a specialized compartment. The appearance of synaptophysin in endosomes of transfected fibroblasts supports the second hypothesis. Our results suggest that synaptophysin may be the key protein contributing endosomal internalization on synaptic vesicle proteins in neurons and endocrine cells, because two other proteins, SV2 and synaptotagmin, do not colocalize with endosomal markers in nonneuroendocrine cells. It will be important to determine if either protein ever enters an endosomal compartment in transfected fibroblasts.

An alternative model of synaptic vesicle recycling has recently emerged (Rindler, 1992). Bennett et al. (1992*a*) characterized large, detergent resistant complexes of synaptic vesicle proteins. If these complexes exist *in vivo*, then synaptic vesicles could be created and retrieved after exocytosis by a selective aggregation mechanism akin to the condensation phenomena thought to sort storage granule contents (Kelly, 1991). Our results do not support such a model. We expressed three of the major synaptic vesicle proteins found in the large, relatively insoluble complexes (SV2, synaptophysin and synaptotagmin) in the same CHO cell line and observed that all three segregated independently. In addition, our preliminary results on rab 3A expressing cell lines suggest that this protein is localized predominantly to the ER, independent of other synaptic vesicle protein expression (Feany, M., and K. M. Buckley, unpublished observation). All three proteins should have an opportunity to associate in the Golgi complex, and at least synaptotagmin and synaptophysin are present in the plasma membrane, where they would again have an opportunity to interact. Of course, aggregation could require the presence of other synaptic vesicle proteins that we did not test (e.g., synaptobrevin or the vacuolar proton pump), other neuroendocrine specific proteins, or posttranslational protein modifications. The selective aggregation model of synaptic vesicle formation is also somewhat inconsistent with the observation that in PC12 cells synaptophysin enters synaptic vesicles only after transport to the cell surface via constitutive secretory vesicles and endosomal sorting (Régner-Vigouroux et al., 1991). It would be of interest to determine if complexes of SV2, synaptotagmin and synaptophysin are created after detergent solubilization of our CHO cell lines using the conditions of Bennett et al. (1992*a*).

Expression of three different synaptic vesicle proteins in CHO fibroblasts has revealed three distinct expression patterns, suggesting that each protein contains unique localization information. Interactions between diverse synaptic vesicle proteins, and perhaps other neural- and endocrine-specific factors, may be critical for targeting of the proteins to synaptic vesicles. For example, synaptophysin may endow the recycling vesicle with its endosomal localization. Coexpression of SV2, synaptotagmin and synaptophysin failed to reveal specific interactions between these three proteins. In addition, none of the three proteins either alone or in combi-

nation is capable of creating a synaptic vesicle-like compartment in nonneuronal cells. Further analysis of these and other cell lines will refine our understanding of the hierarchy of sorting information required to create and maintain the synaptic vesicle.

We thank J. Lippincott-Schwartz, K. Moreman, I. Trowbridge, W. Brown, B. Foellmer, R. Jahn, P. De Camilli, and D. Cutler for their kind gifts of antibodies. We also thank the members of the Buckley lab for many helpful suggestions, and Dr. Jeffrey Boone Miller for advice and encouragement.

This work was supported by the Council for Tobacco Research (grant 2606) and the National Institutes of Health (NINDS grant NS27536).

Received for publication 26 February 1993 and in revised form 12 July 1993.

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