

Colocalization of Synaptophysin with Transferrin Receptors: Implications for Synaptic Vesicle Biogenesis

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Abstract. We have reported previously that the synaptic vesicle (SV) protein synaptophysin, when expressed in fibroblastic CHO cells, accumulates in a population of recycling microvesicles. Based on preliminary immunofluorescence observations, we had suggested that synaptophysin is targeted to the preexisting population of microvesicles that recycle transferrin (Johnston, P. A., P. L. Cameron, H. Stukenbrok, R. Jahn, P. De Camilli, and T. C. Südhof. 1989. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2863–2872). In contrast to our results, another group reported that expression of synaptophysin in cells which normally do not express SV proteins results in the generation of a novel population of microvesicles (Leube, R. E., B. Wiedenmann, and W. W. Franke. 1989. *Cell.* 59:433–446). We report here a series of morphological and biochemical studies conclusively demonstrating that synaptophysin and transferrin receptors are indeed colocalized on the same vesicles in transfected CHO cells. These observations prompted us to investigate whether an overlap between the distribution of the two proteins also occurs in endocrine cell lines that endogenously express synaptophysin and other SV proteins. We have found

that endocrine cell lines contain two pools of membranes positive for synaptophysin and other SV proteins. One of the two pools also contains transferrin receptors and migrates faster during velocity centrifugation. The other pool is devoid of transferrin receptors and corresponds to vesicles with the same sedimentation characteristics as SVs. These findings suggest that in transfected CHO cells and in endocrine cell lines, synaptophysin follows the same endocytic pathway as transferrin receptors but that in endocrine cells, at some point along this pathway, synaptophysin is sorted away from the recycling receptors into a specialized vesicle population. Finally, using immunofluorescent analyses, we found an overlap between the distribution of synaptophysin and transferrin receptors in the dendrites of hippocampal neurons in primary cultures before synapse formation. Axons were enriched in synaptophysin immunoreactivity but did not contain detectable levels of transferrin receptor immunoreactivity. These results suggest that SVs may have evolved from, as well as coexist with, a constitutively recycling vesicular organelle found in all cells.

THE release of classical, nonpeptide neurotransmitters from nerve endings is mediated by synaptic vesicles (SVs),¹ a homogeneously sized vesicle population (Carlson et al., 1978; Huttner et al., 1983) that is concentrated in nerve endings at the presynaptic side of synapses (Peters et al., 1976; De Camilli and Jahn, 1990). Within nerve terminals, SVs undergo an exo-endocytic recycling where at each local cycle they can be reloaded with neurotransmitter content (Ceccarelli et al., 1973; Heuser and Reese, 1973). The recycling of synaptic vesicles appears to involve, at least under certain conditions, an endosome-like compartment (Heuser and Reese, 1973). Thus, an identification of the proteins that participate in SV recycling may pro-

vide important insights into mechanisms that operate during membrane recycling in general.

Until recently, SVs were considered to be a secretory organelle specific to neurons. However, SV polypeptides (synaptotagmin [p65], synaptophysin [p38], synaptobrevin [p18], p29, rab3, and SV2) have been identified by several groups in a variety of peptide hormone secreting endocrine cells (for review see De Camilli and Jahn, 1990). Detailed immunocytochemical and subcellular fractionation analyses indicate that at least some of these polypeptide components are associated with a population of small electron-lucent, pleomorphic vesicles (synaptic-like microvesicles [SLMVs]) that are distinct from the classical peptide-containing secretory granules of endocrine cells (for review see De Camilli and Jahn, 1990). In several studies, synaptophysin (p38), an abundant and well-characterized integral membrane glyco-

1. *Abbreviations used in this paper:* SLMV, synaptic-like microvesicle; SV, synaptic vesicle.

protein of SVs (Jahn et al., 1985; Wiedenmann and Franke, 1985), has proven to be a particularly useful marker protein for the characterization of this vesicle population (Navone et al., 1986; Wiedenmann et al., 1988; Johnston et al., 1989; Clift-O'Grady et al., 1990; Cutler and Cramer, 1990; Schweitzer and Paddock, 1990).

Relationships between SLMVs of endocrine cells and authentic SVs of neurons that extend beyond the observed compositional overlap are becoming apparent. Several pieces of experimental evidence support the hypothesis that SLMVs, like SVs, may undergo an exo-endocytic membrane cycle (Navone et al., 1986; Johnston et al., 1989; Clift-O'Grady et al., 1990). Similarities relevant to 8-aminobutyric acid (GABA) uptake and storage between SLMVs of pancreatic β -cells and SVs of GABAergic neurons suggest that SLMVs and SVs may also be functionally related (Reetz et al., 1991). Thus, a clarification of SLMV biogenesis and membrane traffic may provide insights into the mechanism of SV biogenesis and recycling that cannot be addressed as easily in neurons.

The indications that SLMVs represent an exo-endocytic recycling vesicle population in endocrine cells has raised the possibility that these organelles may be related to the vesicles that recycle plasma membrane components in all cells. To address this issue, one experimental approach taken has been to analyze the fate of synaptophysin when expressed as a foreign protein in nonneuronal, nonendocrine cells. The interpretations of the results obtained to date conflict with each other. We have expressed synaptophysin in CHO cells by cDNA transfection (CHO38 cells) and have shown that it accumulates in the membrane of a population of electron-lucent vesicles which could be labeled by fluid-phase markers of the endocytic pathway (Johnston et al., 1989). On the basis of double-immunofluorescence experiments, we suggested that synaptophysin was targeted to a preexisting vesicle population that constitutively recycles transferrin via the receptor-mediated endocytosis pathway (Johnston et al., 1989). In contrast, other investigators have concluded that synaptophysin containing vesicles identified in transfected cells are induced de novo (Leube et al., 1989). Accordingly, the distinction between the formation of a novel vesicle population as a consequence of synaptophysin expression and the targeting of synaptophysin to a preexisting vesicle population in transfected cells remained a crucial issue to be reconciled.

If synaptophysin expressed as an exogenous protein is targeted to the same vesicle population that recycles transferrin receptors in transfected cells, then the pathway of synaptophysin and transferrin receptors may also be related in cells which endogenously express the protein (endocrine cells and neurons). To address this issue, we have now performed a detailed evaluation of the relationships between synaptophysin containing organelles and transferrin receptors containing organelles in transfected fibroblastic cells and in endocrine cells using a variety of different approaches. We have found that in transfected cells, synaptophysin is localized to the same vesicles that contain transferrin receptors. In two endocrine cell lines, PC12 cells (a cell line derived from a rat pheochromocytoma; Greene and Tischler, 1976) and RINm5F cells (a cell line derived from a rat insulinoma; Gazdar et al., 1980; Bhatena et al., 1982) we have demonstrated that there is a major overlap between

the organelles that contain synaptophysin and those that contain transferrin receptors, with only a fraction of the total synaptophysin segregating into a separate vesicle population. This is in contrast with a recent report showing that synaptophysin and transferrin internalized by receptor mediated endocytosis are not colocalized in PC12 cells (Clift-O'Grady et al., 1990). Additionally, in hippocampal neurons in primary cell cultures a partial co-localization of synaptophysin and transferrin receptors was observed in cell bodies and dendrites, however, transferrin receptors were not detectable in axons where synaptophysin was preferentially concentrated. Together, these results suggest that SVs may have evolved from, as well as coexist with, a constitutively recycling vesicular organelle found in all cells.

Materials and Methods

Primary Antibodies

The following antibodies were generated and characterized as previously described: affinity-purified polyclonal and monoclonal antibodies against synaptophysin (Jahn et al., 1985; Navone et al., 1986), affinity-purified polyclonal antibodies against protein p29 (Baumert et al., 1990), affinity-purified polyclonal antibodies against synaptobrevin (Baumert et al., 1989), and polyclonal antibodies against p65 (Perin et al., 1991). An mAb directed against a cytoplasmic epitope of the human transferrin receptor was a kind gift from Dr. I. Trowbridge (Salk Institute, San Diego, CA), polyclonal antibodies directed against human transferrin were purchased from Axell Accurate Chemical Scientific Corp. (Westbury, NY).

Cell Cultures

CHO-K1 cells were transfected with a full-length cDNA encoding rat synaptophysin as described in Johnston et al. (1989). Cloned cell lines stably expressing synaptophysin (CHO38 cells) were grown in DMEM media supplemented with 10% heat-inactivated FBS, 20 mM Hepes, pH 7.4, 34 μ g/ml proline, 100 U/ml penicillin, and 100 μ g/ml streptomycin and maintained under constant selection with 700 μ g/ml G418 (Gibco/Bethesda Research Laboratories, Gaithersburg, MD) at 37°C in 5% CO₂. PC12 cells (Greene and Tischler, 1976) were grown in RPMI 1640 media supplemented with 10% heat-inactivated horse serum, 5% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in 10% CO₂. RINm5F cells (Gazdar et al., 1980; Bhatena et al., 1982) were grown in RPMI 1640 media supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in 10% CO₂.

Primary cultures of neurons were prepared from the hippocampi of 18 day-old fetal rats as described by Banker and Cowan (1977) and Bartlett and Banker (1984) except that 1% HL1 supplement (Ventrex, Portland, ME) was used in the culture medium instead of the N2 supplement of Bottenstein and Sato (1979) and cortical glial cells were prepared according to the procedure of McCarthy and de Vellis (1980).

Nocodazole Treatment

To depolymerize microtubules, cells were preincubated for 60 min at 37°C in media containing 10 μ g/ml nocodazole (Sigma Chemical Co., St. Louis, MO), diluted from a 10 mg/ml nocodazole stock solution prepared in DMSO (Sigma Chemical Co.). Cells were incubated for an additional 60 min in serum-free media containing 10 μ g/ml nocodazole and 10–20 μ g/ml diferric human transferrin that had been iron saturated following the procedure of Bates and Schlabach (1973). At the end of the transferrin uptake period, the cells were rinsed rapidly in serum-free media, then fixed and processed for immunofluorescent analysis as described below.

Immunofluorescence Labeling

For immunofluorescent analysis, PC12 cells and RINm5F cells were plated onto polyornithine/rat tail collagen-coated glass coverslips, and CHO38 cells were grown on uncoated glass coverslips. Cells were fixed with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.12 M Na phosphate buffer, pH 7.4, washed in the same buffer and incubated with

buffer A (0.5 M NaCl, 20 mM Na phosphate buffer, pH 7.4, 0.3% Triton X-100, and 16% goat serum) to permeabilize cells and quench nonspecific protein binding sites. Cells were then incubated with primary and secondary antibodies (FITC-conjugated goat anti-mouse antibodies [Sigma Chemical Co., St. Louis, MO] and rhodamine-conjugated goat anti-rabbit antibodies [Cappel Organon Teknika, Durham, NC]) diluted in buffer A. Coverslips were then mounted with a freshly prepared solution containing 70% glycerol, 1 mg/ml *p*-phenylenediamine, 150 mM NaCl, 10 mM phosphate buffer, pH 7.4, and then examined using a Zeiss Axiophot microscope equipped with epifluorescence optics and photographed using Kodak T-Max-100 film.

Sucrose Equilibrium Density Gradients

Tissue culture cells were rinsed with homogenization buffer (0.25 M sucrose, 4.0 mM Hepes, pH 7.4, 1 mM MgCl₂) and either resuspended in or scraped into a small volume of homogenization buffer supplemented with protease inhibitor cocktail (1 μg/ml each of leupeptin, aprotinin, pepstatin, and antipain [Sigma Chemical Co., St. Louis, MO], 0.4 mM PMSF [Boehringer Mannheim Biochemicals, Indianapolis, IN], with or without 0.005% DNase [Sigma Chemical Co.]). Homogenization was routinely performed by passing cells through a ball bearing cell cracker (Bernie-tech Engineering, Saratoga, CA) with a 0.0012-in. clearance, although similar results were obtained using a Dounce homogenizer. The cell lysate was centrifuged for 15 min at 676 *g*_{av} in a Hill microfuge (Hill Scientific Instruments, Derby, CT). The pellet was resuspended in 1 ml of supplemented homogenization medium, passed twice through the cell cracker and spun as before. The two low speed supernatants were combined, adjusted to 1.2 mM EDTA and layered on top of a 0.35–2.0 M sucrose gradient supplemented with 4.0 mM Hepes, pH 7.4, and 1.2 mM EDTA. Similar results were obtained when the sample was loaded at the bottom of the sucrose gradient. Gradients were spun using a SW41 rotor (Beckman Instruments Inc., Palo Alto, CA) for 20 h at 25,000 rpm (4°C), after which 1-ml fractions were collected from the top of the centrifuge tubes. The refractive indexes of all samples were read and sucrose densities determined to confirm that all gradients were linear.

Organelle Immunoisolation

Nonimmune bovine immunoglobulins (Sigma Chemical Co., St. Louis, MO), or purified mAb C7.2 directed against synaptophysin were conjugated to Eupergit CLZ methacrylate microbeads (Rohm Pharm, Darmstadt, Germany) as described (Burger et al., 1989). For immunoadsorptions, aliquots of various supernatant fractions (i.e., low speed supernatant and gradient fractions as described above) were incubated with immunobeads for 1–2 h at 4°C with constant nutation. The beads were sedimented by centrifugation for 30–60 s in a Hill microfuge and then washed four to six times, alternating between homogenization buffer and a solution containing 300 mM NaCl, 20 mM Na phosphate, pH 7.4. For morphological analyses, an excess of the starting fraction was used to ensure maximum binding or organelles to the immunobeads. For biochemical analyses an excess of antisynaptophysin immunobeads was used to ensure quantitative immunoadsorption of all the synaptophysin from the starting material (determined for each batch of immunobeads).

Electron Microscopy of Immunisolated Organelles

Immunobead samples were fixed in 2.5% glutaraldehyde, 0.1 M Na cacodylate, pH 7.4, on ice, rinsed in 0.1 M Na cacodylate buffer, pH 7.4, and then embedded in agarose according to the procedure of De Camilli et al. (1983). Agarose squares were washed with cacodylate buffer, post-fixed with 1% OsO₄ in the same buffer, stained en bloc using A-V buffer containing 0.5% uranyl magnesium acetate, dehydrated, and finally embedded in a mixture of Epon 812/Araldite 6005/DDSA/2% DMP-30. Ultrathin sections were counterstained with 4.0% uranyl acetate and 0.2% lead citrate and analyzed using a Phillips 301 electron microscope.

Immunogold Labeling of Immunisolated Organelles

Immunobead samples were fixed in 3% formaldehyde–0.25% glutaraldehyde in 0.25 M sucrose, 7.5 mM Na phosphate buffer, pH 7.4, washed in buffer and embedded in agarose (see above). Agarose squares were washed with 50 mM Tris, pH 7.4, incubated with 0.5 M NaCl, 20 mM Na phosphate buffer, pH 7.4, containing 5% BSA and then immunolabeled with affinity-purified anti-synaptophysin polyclonal antibodies followed by protein A–gold conjugates prepared according to the procedure of Slot and Geuze

(1985). Samples were postfixed with 1% glutaraldehyde and with 1% OsO₄ and further processed as described above.

Glycerol Velocity Gradients

Glycerol velocity gradients were performed as described by Clift-O'Grady et al. (1990). Cells were homogenized as described above for sucrose equilibrium density gradients with the exception that the homogenization medium was 150 mM NaCl, 10 mM Hepes, pH 7.4, 1 mM EGTA, 0.1 mM MgCl₂, supplemented with protease inhibitor cocktail and DNase as described. The low-speed supernatant was loaded onto a 5–25% continuous glycerol gradient with a 50% sucrose cushion. Gradient fractions (1 ml each) were collected from the top of the centrifuge tubes.

Electrophoresis and Immunoblot Analysis

SDS-PAGE was performed according to Laemmli et al. (1970). Western blotting was performed as described by Towbin et al. (1979) using either ¹²⁵I-protein A (Amersham Chemical Co., Arlington Heights, IL) or the enhanced chemiluminescence (ECL) kit (Amersham Chemical Co.).

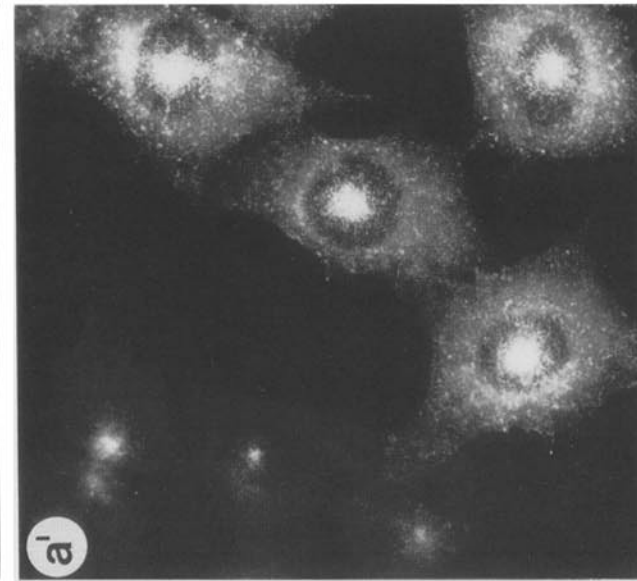
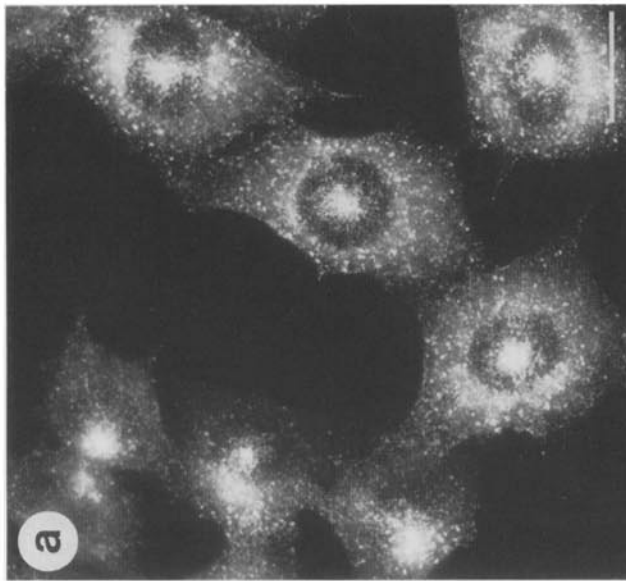
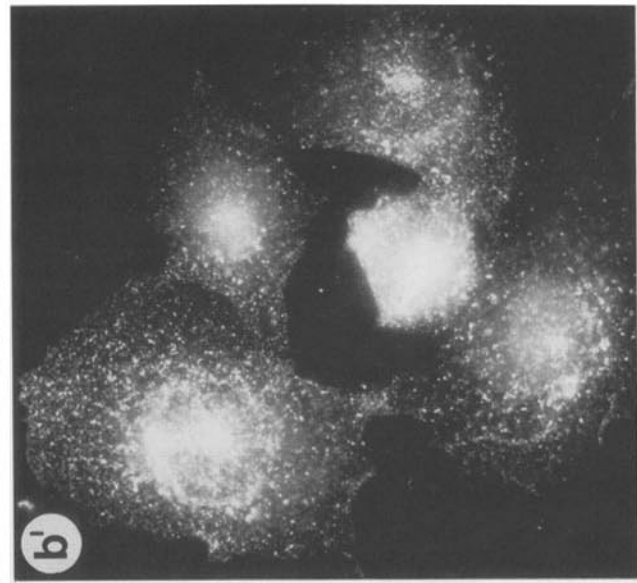
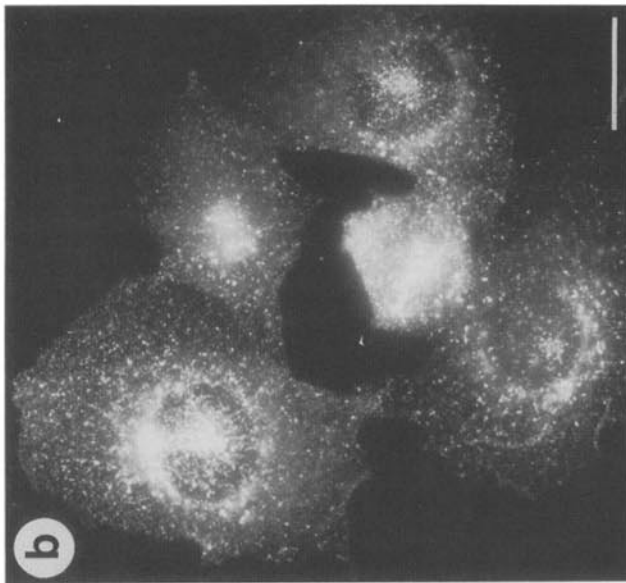
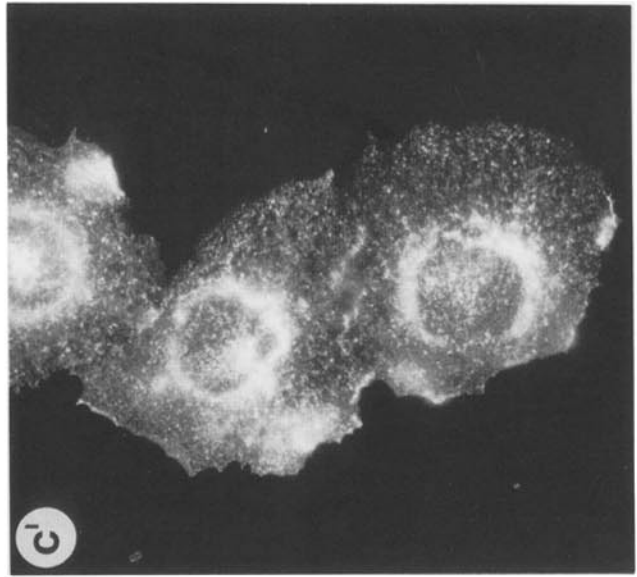
Results

Synaptophysin and Transferrin Receptors Are Localized in the Same Vesicle Population in CHO38 Cells

We carried out a detailed investigation of the relationships between the vesicles that accumulate synaptophysin and the vesicles that recycle transferrin receptors using a clone of transfected CHO cells isolated previously, CHO38 cells (Johnston et al., 1989). In agreement with preliminary observations (Johnston et al., 1989), CHO38 cells incubated in the presence of exogenously added iron-saturated transferrin and processed for double-labeling immunofluorescent analysis showed a virtually identical staining pattern for synaptophysin and transferrin (Fig. 1, *a* and *a'*). The immunofluorescent staining pattern obtained for both proteins is characterized by fine puncta scattered throughout the cell, although they are concentrated particularly in the perinuclear region and above the nucleus. Both of these perinuclear and epinuclear localization patterns correspond to sites from which microtubules originate (Fig. 2 *a'*).

The concentration of transferrin used for these uptake experiments was aimed at optimizing receptor mediated, rather than bulk fluid, uptake. Consequently, the localization of transferrin is likely to reflect the distribution of transferrin receptors since transferrin has been shown to remain bound to its receptor during recycling (Klausner et al., 1983; Dautry-Varsat et al., 1983). After double labeling CHO38 cells for transferrin and for transferrin receptors (Fig. 1, *b* and *b'*), identical immunostaining patterns were observed; thereby confirming that either the membrane receptor or the soluble ligand can be used to identify the same pathway under the experimental conditions employed. Accordingly, a nearly identical immunostaining pattern was observed after double labeling for synaptophysin and for transferrin receptors (Fig. 1, *c* and *c'*). Similar immunolocalization analysis of internalized transferrin and transferrin receptors carried out in mock-transfected CHO cells (i.e., transfected with the neomycin-resistant gene only; Johnston et al., 1989) indicated that the expression of synaptophysin did not alter the normal cellular distribution of transferrin or transferrin receptors.

If synaptophysin and transferrin receptors are residents of the same vesicle population, then alterations of the distribu-



tion of vesicles containing one protein should concomitantly change the distribution of the other protein. The intracellular redistribution of transferrin receptor containing organelles in cells treated with the drug nocodazole, which induces the disassembly of microtubules, has been reported previously (De Brabander et al., 1988). Therefore, we determined whether nocodazole mediated depolymerization of microtubules would lead to a similar redistribution of the synaptophysin and transferrin receptors. CHO38 cells immunolabeled for β -tubulin (to identify microtubules) and for internalized transferrin before and after nocodazole treatment are shown in Fig. 2, *a*, *a'*, *b*, and *b'*. Untreated control cells show the microtubule network and the characteristic accumulation of transferrin immunoreactivity at microtubule-organizing centers (Yamashiro et al., 1984) (Fig. 2, *a* and *a'*). Cells pretreated with nocodazole and subsequently supplied with exogenous transferrin in the continued presence of the drug are shown in Fig. 2, *b*, *b' c*, and *c'*. As indicated by the diffuse staining pattern obtained for β -tubulin (Fig. 2 *b'*), nocodazole treatment produced a complete depolymerization of the microtubular network. Although receptor-mediated endocytosis of transferrin continued in the presence of nocodazole, vesicles containing transferrin immunoreactivity appeared scattered throughout the cytoplasm and were not accumulated characteristically in the perinuclear region (Fig. 2, *b* and *c'*). Additionally, accumulations of transferrin immunoreactivity could be observed at the cell periphery, especially at contact regions between adjacent cells (Fig. 2, *b* and *c'*). As anticipated, the immunofluorescent pattern detected for synaptophysin and transferrin overlapped even after nocodazole treatment of the CHO38 cells (Fig. 2, *c* and *c'*). Together, these data are consistent with the suggestion that a significant amount of the synaptophysin expressed in CHO38 cells is localized to the vesicles that recycle transferrin receptors.

Organelle immunoisolation experiments were carried out as a means to provide a more conclusive demonstration that synaptophysin and transferrin receptors were residents of the same vesicles. For this purpose, mAbs directed against the cytoplasmic tail of synaptophysin were covalently conjugated to methacrylate beads (anti-synaptophysin immunobeads) and subsequently used to immunoisolate synaptophysin containing organelles (Burger et al., 1989).

Postnuclear supernatants prepared from CHO38 cell homogenates were used for immunoisolations either directly or after being fractionated on sucrose-density equilibrium gradients. Immunoblot analysis of fractions obtained by equilibrium sedimentation in continuous sucrose gradients revealed that transferrin receptors and synaptophysin sedimented to similar densities (data not shown). Transferrin receptors sedimented to the same density regardless of whether the load fraction was prepared from CHO38 cells or from mock-trans-

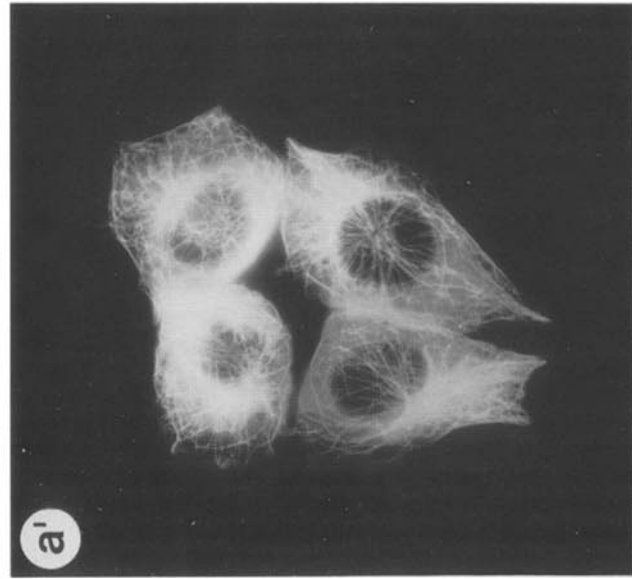
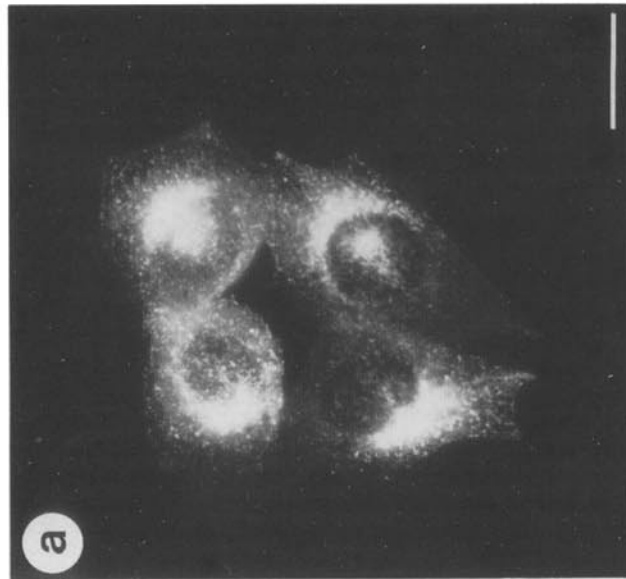
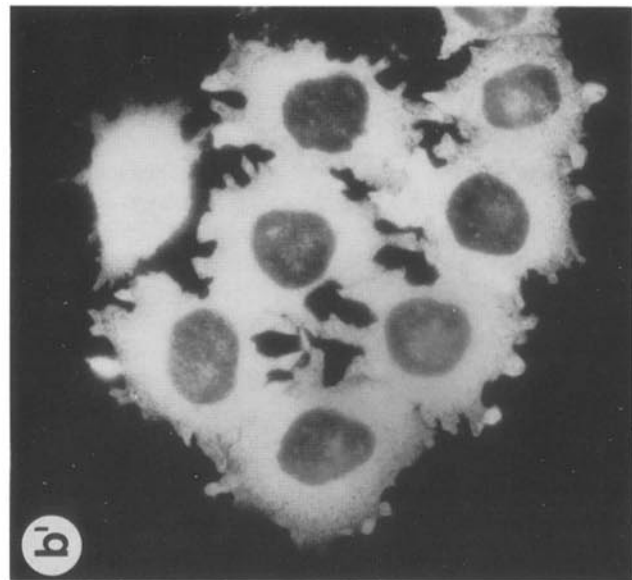
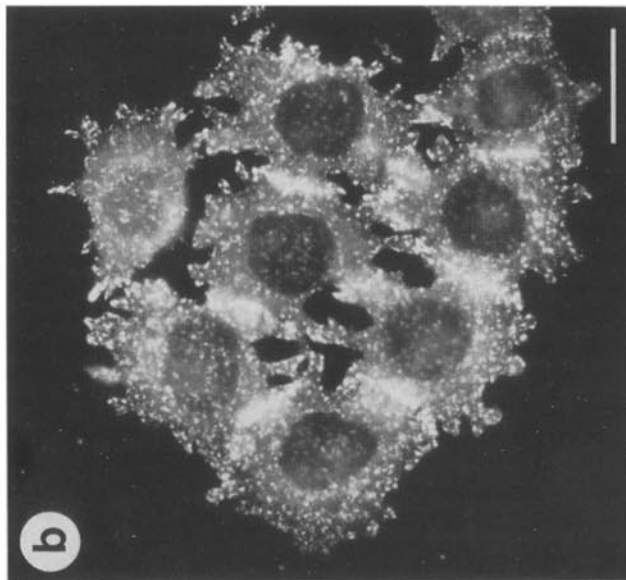
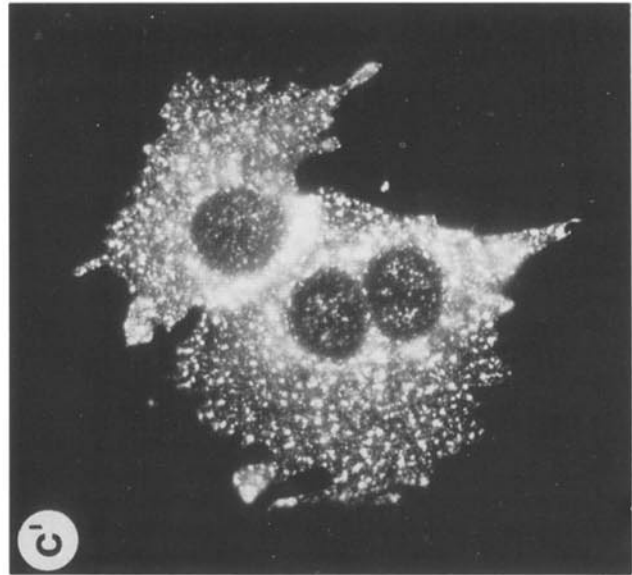
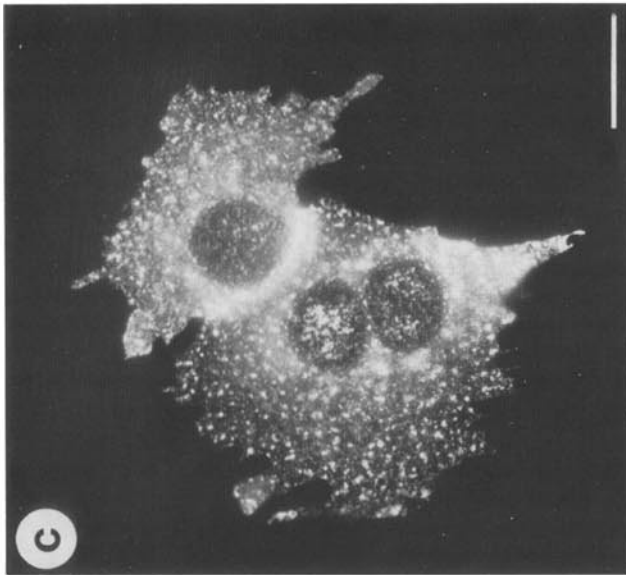
fected CHO cells. As shown in Fig. 3 *A*, gradient fractions 4 and 5 (corresponding to the densities of 1.094 and 1.108 g/cm³, respectively), contained the greatest amount of synaptophysin and were therefore used for immunoisolations. The immunoblots of Fig. 3 *B* show that synaptophysin was depleted quantitatively from the starting material and subsequently recovered in the washed bead fraction. In addition, transferrin receptors were recovered predominately in the synaptophysin-enriched fractions. Similar results could be obtained when unfractionated postnuclear supernatants were used for the immunoisolation. As shown in Fig. 4 *A*, synaptophysin and the majority of transferrin receptors were depleted directly from the postnuclear supernatant and recovered in the immunoadsorbed fraction. Neither synaptophysin nor transferrin receptors were recovered in the bead fraction when immunoadsorptions were performed using methacrylate beads coated with nonimmune bovine IgGs (Fig. 4 *B*).

The morphology of the organelles immunoisolated from a postnuclear supernatant prepared from CHO38 cells and mock-transfected CHO cells was assessed by electron microscopy. As shown in Fig. 5, only anti-synaptophysin immunobeads incubated with extracts prepared from cells expressing synaptophysin are coated by organelles. The immunoisolated organelles are represented principally by pleomorphic, small vesicles and tubules (Fig. 5 *a*). Also a few larger, irregularly shaped profiles were observed. These results are consistent with the morphology of synaptophysin-positive structures previously observed in CHO38 cells using electron microscopic immunocytochemical analyses (Johnston et al., 1989). No organelles were seen on immunobeads after incubation with a postnuclear supernatant prepared from the mock-transfected CHO cells (Fig. 5 *b*). In addition, no organelles were seen on immunobeads coated with non-immune bovine IgGs after incubation with supernatants prepared from CHO38 cells (data not shown).

As an additional way to determine the extent of overlap between synaptophysin and transferrin receptors, a low speed supernatant prepared from a CHO38 cell homogenate was analyzed by velocity sedimentation in continuous glycerol gradients with a sucrose cushion at the bottom as described by Clift-O'Grady et al. (1990). These gradients offer the possibility to resolve organelles that have the same buoyant density but different sedimentation coefficients. Immunoblot analyses of gradient fractions revealed a similar sedimentation profile for synaptophysin and transferrin receptors (Fig. 6). Both proteins become progressively more enriched towards the bottom fractions although the amount of synaptophysin compared to transferrin receptors may be slightly higher in the upper portion of the gradient.

Together, our results demonstrate that synaptophysin, when expressed in transfected CHO cells, is localized primarily to the vesicles that recycle transferrin receptors.

Figure 1. Comparison of the distribution of synaptophysin, internalized transferrin, and transferrin receptors in CHO38 cells by immunofluorescence. Cells were double-labeled for the three proteins as follows: (*a*, *a'*) internalized transferrin and synaptophysin, respectively; (*b*, *b'*) transferrin receptors and internalized transferrin, respectively; (*c*, *c'*) transferrin receptors and synaptophysin, respectively. CHO38 cells shown in *a*, *a'* and *b*, *b'* were incubated in the presence of 10–20 μ g/ml human transferrin for 60 min before fixation. The localization pattern for all three proteins is characterized by small immunoreactive puncta, which are particularly concentrated around and over the nucleus. Note the striking similarity within each pair of micrographs. The difference seen in the pair *a*, *a'* is due to low levels of synaptophysin expression in the three cells in the top left corner. Bars: (*a*, *a'*) 34 μ m; (*b*, *b'*, *c*, *c'*) 22 μ m.



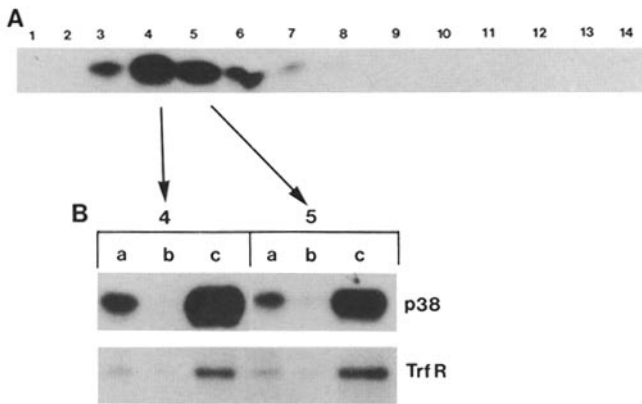


Figure 3. Immunoisolation of synaptophysin and transferrin receptor containing organelles from sucrose equilibrium gradient fractions of CHO38 cells. A postnuclear supernatant (676 g_w) obtained from CHO38 cell homogenates was layered on top of a 0.35–2.0 M continuous sucrose gradient and sedimented to equilibrium density. Fraction 1 corresponds to the top of the gradient. Equal volumes of each gradient fraction were separated by SDS-PAGE, transferred electrophoretically to nitrocellulose, immunolabeled with antibodies directed against synaptophysin and detected with ^{125}I -protein A. Immunoblot analyses of collected gradient fractions (A) shows that synaptophysin is enriched in fractions 3–6. Aliquots from gradient fractions 4 (0.72 M sucrose) and 5 (0.82 M sucrose) were incubated with anti-synaptophysin immunobeads. B shows immunoblot analyses of the same volumes of the gradient fractions before (lanes a) and after (lanes b) immunoisolation. Lanes c show the immunoisolated material recovered from ~ 10 times the amount of starting material shown in a. Both synaptophysin (p38) and transferrin receptors (TrfR) are recovered in the immunoisolated material (c).

A Pool of Synaptophysin Is Localized in the Same Vesicle Population as Transferrin Receptors in Endocrine-derived Cell Lines

We have shown previously that the synaptophysin-positive vesicles in CHO38 cells share many similarities with the SLMVs of undifferentiated PC12 cells (a prototypic endocrine tissue culture cell line). These similarities include morphology, intracellular distribution, and the property to become labeled by extracellular tracers (Johnston et al., 1989). Consequently, we pursued the question of whether an overlap exists in endocrine cells between SLMVs and the vesicles that recycle transferrin receptors.

PC12 cells, immunolabeled for internalized transferrin and for synaptophysin, and RINm5F cells immunolabeled

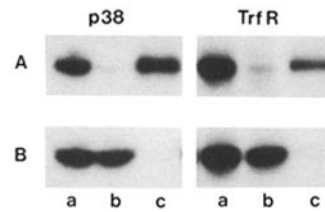


Figure 4. Immunoblot analyses of organelles immunoisolated from CHO38 cells. A postnuclear supernatant prepared from a CHO38 cell homogenate was incubated with either anti-synaptophysin immunobeads (A) or control immunobeads coated with nonimmune IgGs (B). Immunoblot analyses of the same volumes of the postnuclear supernatant before (lanes a) and after (lanes b) immunoisolation and of the immunisolated material recovered from a same amount of postnuclear supernatant (lanes c) are shown. Virtually all synaptophysin (p38) and the majority of transferrin receptor (TrfR) were removed from the starting material and recovered in the immunobead fraction only when anti-synaptophysin immunobeads were used.

for transferrin receptors and for synaptophysin are shown in Fig. 7, a, a' and b, b', respectively. Although distinctions in fine details can be observed, the overall immunofluorescent staining pattern for synaptophysin and for transferrin/transferrin receptors is very similar. As shown for CHO38 cells (Fig. 1, b and b'), identical immunolocalization patterns were produced in PC12 cells and RINm5F cells by immunolabeling either for transferrin receptors or for transferrin internalized by receptor mediated endocytosis (data not shown).

Subsequently, we investigated whether transferrin receptors could be detected as components of synaptophysin-positive organelles that were obtained by subcellular fractionation. Immunoisolation experiments, analogous to those described for CHO38 cells, were performed using a postnuclear supernatant prepared from a PC12 cell homogenate. As shown in Fig. 8 A, synaptophysin was depleted from the starting material and quantitatively recovered in the immunobead fraction. The depletion of synaptophysin from the low-speed supernatant was paralleled by a major depletion of the SV protein p29 (Fig. 8 C), a finding consistent with the previous demonstration that synaptophysin-containing vesicles of PC12 cells comprise additional SV polypeptides (Baumert et al., 1990). Significantly, the immunoadsorbed fraction also contains the majority of the transferrin receptor population (Fig. 8 B). Neither synaptophysin, p29, nor transferrin receptors were found in the immunoadsorbed fraction when anti-synaptophysin immunobeads were substituted with beads coated with non-immune bovine IgGs (Fig. 8 D and not shown).

The morphological appearance of organelles immunoisolated from PC12 cells is shown in Fig. 9. These organelles

Figure 2. Redistribution of internalized transferrin and of synaptophysin induced by the microtubule-depolymerizing drug nocodazole. CHO38 cells were double-labeled for the following proteins: (a, a') internalized transferrin and β -tubulin, respectively; (b, b') internalized transferrin and β -tubulin, respectively; (c, c') synaptophysin and internalized transferrin, respectively. Before fixation, cells shown in b, b' and c, c' were incubated in the presence of nocodazole for 60 min, then allowed to internalize exogenously added transferrin for an additional 60 min in the continued presence of the drug as described in Materials and Methods. The control cells shown in a, a' were processed in parallel but without the addition of nocodazole. After nocodazole treatment, microtubules are depolymerized as assessed by the diffuse immunofluorescence pattern observed for β -tubulin (compare a' with b'). In control cells puncta immunoreactive for internalized transferrin are concentrated at microtubule-organizing centers. In nocodazole-treated cells puncta immunoreactive for internalized transferrin and for synaptophysin are scattered throughout the cells. The colocalization of synaptophysin and internalized transferrin is maintained after microtubule depolymerization. Bars: (a, a', c, c') 20 μ m; (b, b') 24 μ m.

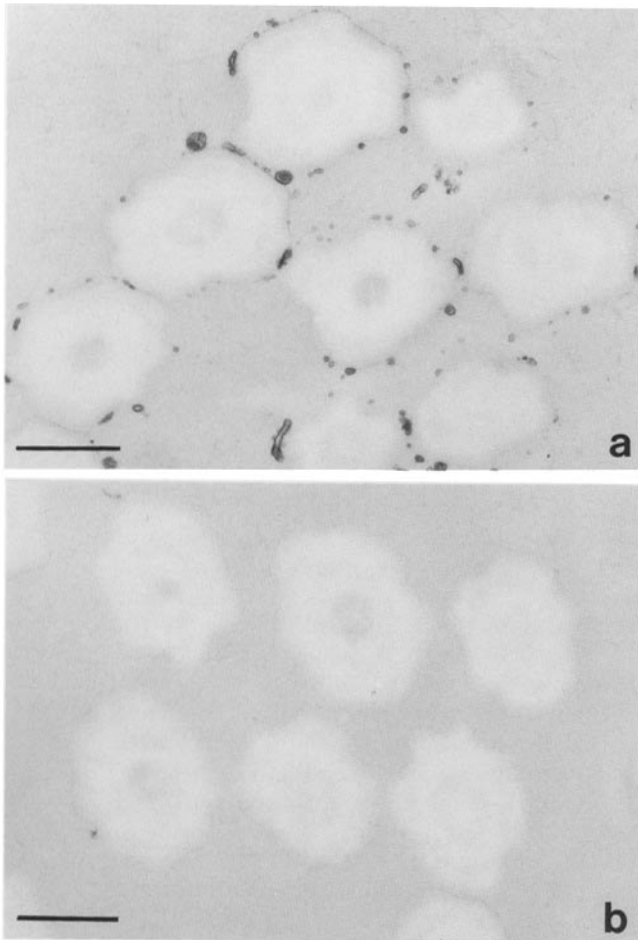


Figure 5. Electron microscopic analyses of organelles immunoprecipitated from CHO cells. Anti-synaptophysin immunobeads were incubated with a postnuclear supernatant obtained from CHO38 cells (a) or from CHO cells mock transfected with the neomycin resistant gene alone (b). Organelles immunoprecipitated to the beads are represented, for the most part, by small vesicles and tubules. A few larger vacuolar structures are also visible. No organelles are visible on the beads shown in b. Bars, 0.66 μm .

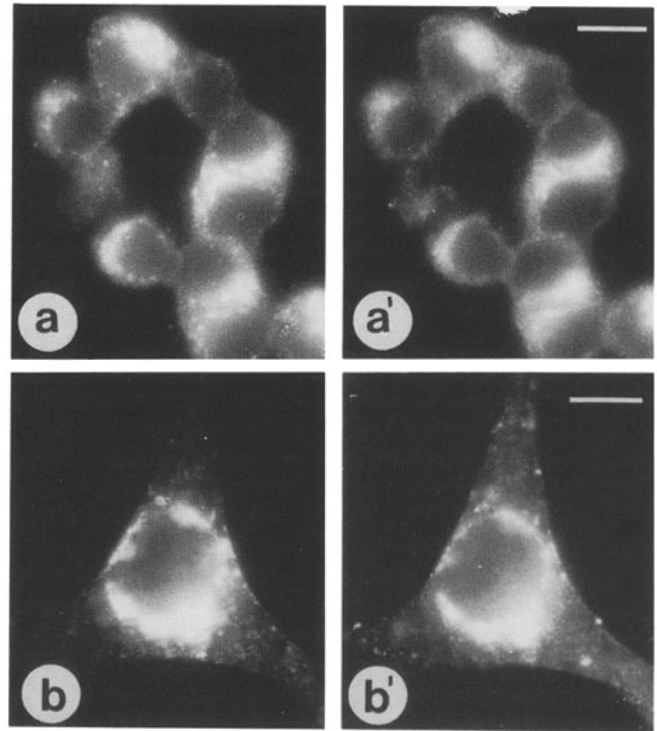


Figure 7. Comparison of the distribution of synaptophysin with internalized transferrin in PC12 cells and with transferrin receptors in RINm5F cells by immunofluorescence. (a, a') Undifferentiated PC12 cells were incubated for 60 min in the presence of 10–20 $\mu\text{g/ml}$ exogenously added human transferrin before fixation and then double-labeled for internalized transferrin (a) and for synaptophysin (a'). (b, b') Double-labeling for transferrin receptors (b) synaptophysin (b') in RINm5F cells. In general, both synaptophysin immunoreactivity and transferrin/transferrin receptor immunoreactivities are represented by small puncta scattered throughout the cytoplasm but particularly concentrated in the perinuclear region. Bars, 8.5 μm .

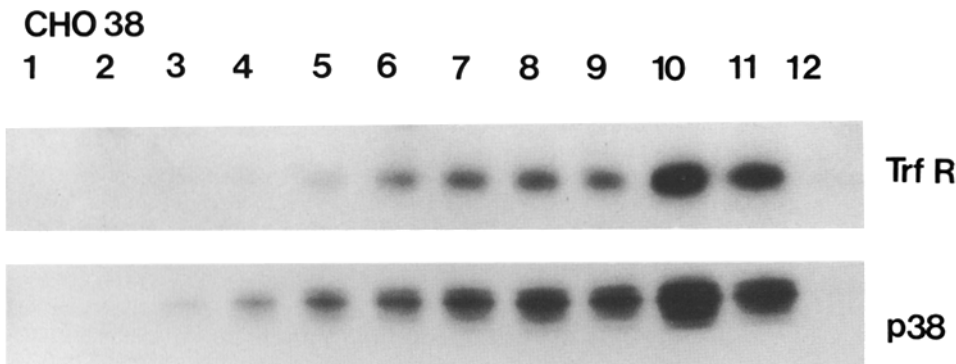


Figure 6. Distribution of synaptophysin and transferrin receptors in glycerol velocity gradients. A low-speed supernatant prepared from homogenates of CHO38 cells was separated by velocity centrifugation on a 5–25% glycerol gradient with a 50% sucrose cushion at the bottom. Fractions were collected from the top of the gradient (fraction 1 corresponds to the top of the gradient) and equal volumes of each gradient fraction were separated by SDS-PAGE electrophoresis. The figure shows immunoblot analysis of the gradient fractions demonstrating an overlapping distribution of synaptophysin (p38) and transferrin receptors (TrfR).

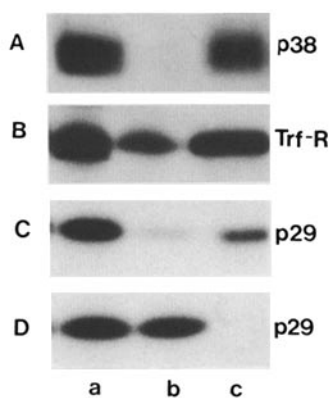


Figure 8. Immunoblot analyses of organelles immunoisolated from PC12 cells. A post-nuclear supernatant prepared from a PC12 cell homogenate was incubated with either anti-synaptophysin immunobeads (A-C) or control immunobeads coated with nonimmune IgGs (D). Immunoblot analysis of the same volumes of the post-nuclear supernatant before (lane *a*) and after (lane *b*) immunoisolation and of the immunoisolated material recovered from a same amount of

postnuclear supernatant (lane *c*) are shown. The depletion of synaptophysin (*p38*) from the starting material was accompanied by a significant removal of transferrin receptors (*TrfR*) from the starting material. Another SV protein, *p29* was recovered in the immunobead fraction only when anti-synaptophysin immunobeads were used (C) but not when control immunobeads coated with nonimmune bovine IgGs were used (D).

are similar in morphology to the synaptophysin-positive organelles of endocrine cells as determined by electron microscopic immunocytochemical analysis of intact cells (Navone et al., 1986; Johnston et al., 1989). For the most part, they are represented by vesicles and tubules of variable size and shape (Fig. 9, *a* and *b*). Confirming that the immunoisolation is specific for synaptophysin-containing organelles, all of the organelles bound to the beads were immunoreactive for syn-

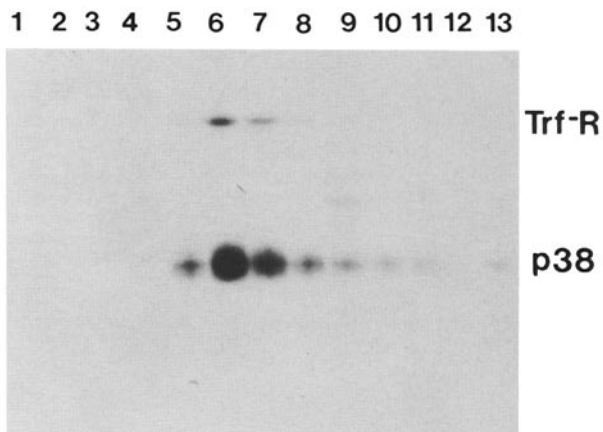


Figure 10. Distribution of synaptophysin and transferrin receptors in sucrose equilibrium gradients. A postnuclear supernatant obtained from a PC12 cell homogenate was layered on top of a 0.35–2.0 M continuous sucrose gradient and sedimented to equilibrium density. Fraction 1 corresponds to the top of the gradient. Equal volumes of each gradient fraction were separated by SDS-PAGE. The figure shows immunoblot analysis of the gradient fractions demonstrating that synaptophysin (*p38*) and transferrin receptors (*TrfR*) peak at 0.85 M sucrose (fraction 6) and 0.99 M sucrose (fraction 7).

aptophysin when labeled with an affinity-purified anti-synaptophysin polyclonal antibody followed by protein A-gold (Fig. 9 *b*). Additional specificity of the immunoadsorption procedure was demonstrated by the absence of any organelles at the bead surface when control beads coated with non-

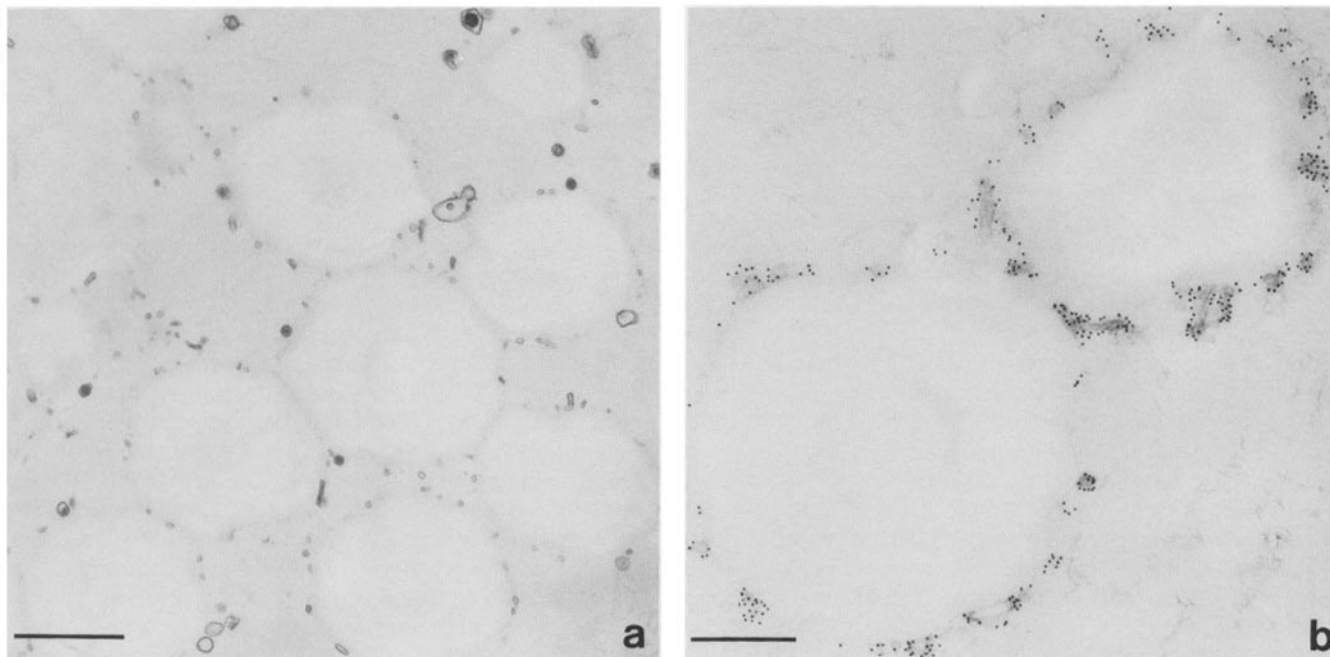


Figure 9. Electron microscopic analyses of organelles immunoisolated from PC12 cells. Organelles immunoadsorbed to the anti-synaptophysin immunobeads are represented, for the most part, by small vesicles and tubules (*a*). A few larger vacuolar structures are also visible. (*b*) Organelles bound to anti-synaptophysin immunobeads were immunolabeled using affinity purified anti-synaptophysin antibodies followed by protein A-gold conjugates. All organelles bound to the immunobeads are shown to be positive for synaptophysin. Bars: (*a*) 0.66 μm ; (*b*) 0.32 μm .

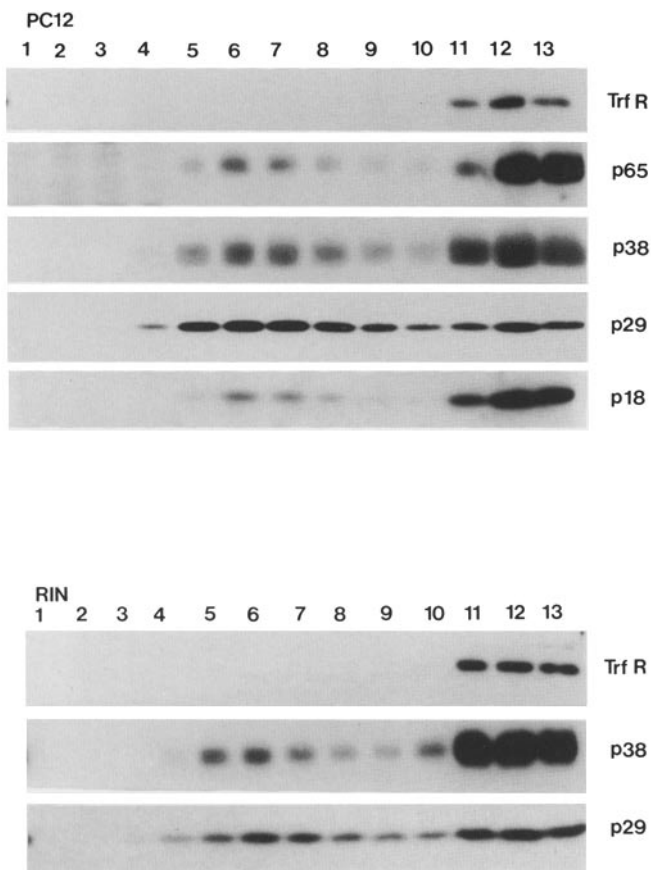


Figure 11. Distribution of synaptophysin and transferrin receptors in glycerol velocity gradients. Low-speed supernatants prepared from homogenates of PC12 or RINm5F cells were separated by velocity centrifugation on a 5–25% glycerol gradient with a 50% sucrose cushion at the bottom. Fraction 1 corresponds to the top of the gradient. Equal volumes of each gradient fraction were separated by SDS-PAGE. Immunoblot analyses of the gradient fractions show two peaks of synaptophysin (*p38*) and of SV proteins (*p65/synaptotagmin*, *p29*, and *p18/synaptobrevin*). Only at the bottom of the gradient is there an overlap of SV proteins with the single peak of transferrin receptors (*TrfR*).

immune bovine IgGs were used for the immunoisolation (data not shown).

To determine if a population of synaptophysin containing organelles that lack transferrin receptor is present in endocrine cells, we compared the distribution of these two proteins by equilibrium and velocity sedimentation. When a postnuclear supernatant obtained from PC12 cells was analyzed by equilibrium sedimentation in continuous sucrose gradients, synaptophysin and transferrin receptors peaked at similar densities (1.108–1.127 g/cm³; Fig. 10). In contrast when low speed supernatants prepared from homogenates of PC12 or RINm5F cells were analyzed by velocity sedimentation in glycerol gradients, a partial dissociation of synaptophysin and transferrin receptor containing membranes was revealed by immunoblot analyses of the collected gradient fractions (Fig. 11). Whereas the majority of synaptophysin peaked together with transferrin receptors at the bottom of the glycerol gradient, a smaller peak of synaptophysin was apparent near the middle of the gradient and did not include any detectable transferrin receptor. In agreement with the

described compositional overlap between SVs and SLMVs, a number of other SV proteins (*p18/synaptobrevin* [Baumert et al., 1989]; *p29* [Baumert et al., 1990]; and *p65/synaptotagmin* [Matthew et al., 1981; Perin et al., 1990]) cosedimented with synaptophysin in these glycerol gradients (Fig. 11). Since all synaptophysin-positive organelles produced a single peak after equilibrium sedimentation on sucrose gradients, the two peaks of SV proteins resolved by velocity sedimentation in glycerol gradients are likely to be explained by vesicles of different sizes.

Together, our morphological and biochemical results indicate a partial overlap in the subcellular distribution of synaptophysin and other SV proteins with transferrin receptors in undifferentiated PC12 cells and in RINm5F cells. It appears that at least two populations of synaptophysin-containing organelles are present in endocrine cell line: one population comprising larger sized organelles that contains transferrin receptors and one population comprising smaller sized organelles that does not.

A Pool of Synaptophysin Is Colocalized with Transferrin Receptors in Developing Neurons in Primary Cultures

Transferrin receptors have not been detected in synaptic vesicles in nerve terminals (our unpublished observations). Accordingly, mature neurons appear to have the biochemical machinery necessary to segregate efficiently synaptophysin from transferrin receptors. To assess the extent to which the segregation of synaptophysin from transferrin receptors is a function of the generation of axons and dendrites, we analyzed the distribution of these two proteins in primary neuronal cell cultures prepared from embryonic rat hippocampi. Hippocampal neurons grown in culture have been characterized extensively and represent a model system to study neuronal cell development and the establishment of polarity of membrane domains (Dotti et al., 1988; Fletcher et al., 1991).

The immunofluorescent staining patterns of synaptophysin and of transferrin receptors were compared in stage 4 (according to the stage designation of Dotti et al., 1988) hippocampal neurons. Dendrites and axons can be distinguished according to morphological criteria: dendrites are shorter and tend to taper in a proximo-distal direction, whereas axons are longer, highly branched with the apparent cross sectional area of the axonal arbor increasing in a proximo-distal gradient. A punctate pattern of synaptophysin immunoreactivity could be detected throughout the cell. However, in agreement with Fletcher et al. (1991), synaptophysin immunoreactivity was observed to be enriched primarily in axonal compartments (Fig. 12 *a*). In contrast, transferrin receptor immunoreactivity was not detectable in the axon but was concentrated in the dendrites and the cell body (Fig. 12 *a'*), although in dendrites the intensity of transferrin receptor immunoreactivity was variable. When dendrites were observed at higher magnification, a partial overlap between synaptophysin and transferrin receptor immunoreactivity could be observed.

Discussion

In this study, we have investigated the relationship between the pathway that recycles SV proteins and the receptor-

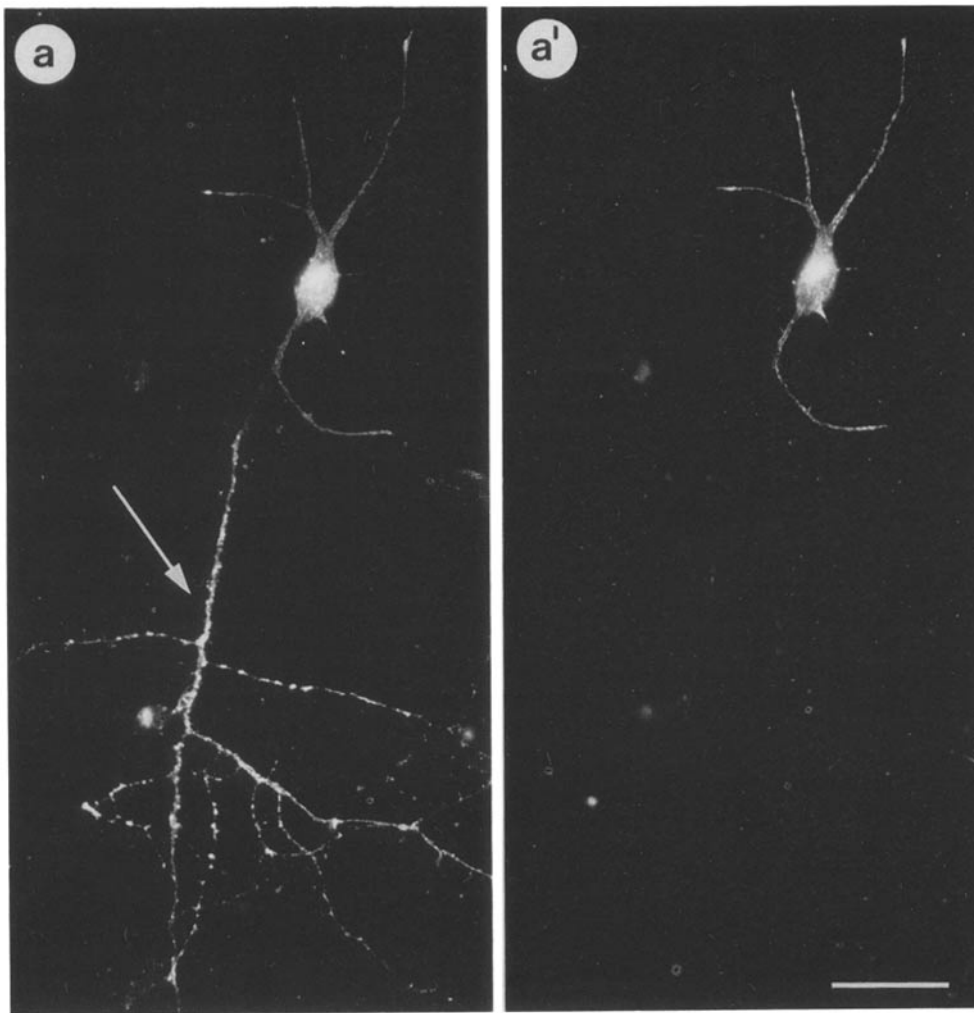


Figure 12. Comparison of the distribution of synaptophysin and transferrin receptors in a cultured hippocampal neuron (stage 4 neuron according to the stage designation of Dotti et al., 1988) by immunofluorescence. Synaptophysin immunoreactivity can be seen throughout the neuron but is enriched in the axon (*arrow*) (*a*). Transferrin receptor immunoreactivity can be seen only in the dendrites and in the cell body but not in the axon (*a'*). Bar, 32 μm .

mediated endocytic pathway (Goldstein and Brown, 1985; Geuze et al., 1986). For this purpose we have compared the distribution of transferrin receptors and of synaptophysin in cells that express synaptophysin endogenously (endocrine cells and neurons) or as a result of cDNA transfection (CHO38 cells). In CHO38 cells, synaptophysin and transferrin receptors were found to be residents of the same vesicle population. In addition, a partial overlap in the subcellular distribution of the two proteins was observed in endocrine cell lines and in primary neuronal cell cultures. These findings suggest that SLMVs and SVs represent a special adaptation of an exo-endocytic recycling pathway which takes place in all cells.

In CHO38 cells, a virtually identical subcellular localization of synaptophysin and transferrin receptors was demonstrated by a variety of complementary procedures including double immunofluorescence, sedimentation in density, and velocity gradients as well as organelle immunoisolation. The incomplete recovery of transferrin receptors on anti-synaptophysin immunobeads (Fig. 4) may reflect the presence of CHO38 cells that do not express synaptophysin. However, we cannot exclude the possibility that a minor subpopulation of organelles is present in our CHO38 cell cultures that contain transferrin receptors but not synaptophysin. Our results on the colocalization of synaptophysin with transferrin re-

ceptors in CHO38 cells validate this cell line as a useful model system to study the mechanisms of recycling of synaptophysin.

The present findings indicate that synaptophysin when expressed in CHO38 cells is targeted to a preexisting vesicle population. Leube et al. (1989) also have expressed synaptophysin in a variety of nonneuronal, nonendocrine cells. However, in contrast to our conclusion they suggested that synaptophysin acts as a vesicle organizer, i.e., that the protein contains sufficient information to sort itself into a special vesicle in the absence of other SV proteins. Although the distribution of synaptophysin in their transfected cells was similar to what we observed, they did not compare the distribution of synaptophysin with that of any recycling plasma-membral receptor.

Our observation that synaptophysin and transferrin receptors are colocalized in the same vesicle population in CHO38 cells prompted us to compare the distribution of the two proteins in cells that express synaptophysin endogenously. The results we have described for PC12 and RINm5F cells suggest that in these endocrine cell lines, the recycling pathways of synaptophysin and of transferrin receptors are interconnected, to a large extent. Immunolocalization studies revealed a significant overlap of synaptophysin and transferrin/transferrin receptors. Immunoisolation experiments

demonstrated that the majority of transferrin receptors could be recovered with synaptophysin. Fractionation on glycerol velocity gradients of low-speed supernatants obtained from these endocrine cell lines, revealed two peaks of synaptophysin along with other SV proteins. One peak was devoid of transferrin receptors and is likely to correspond to an analogous peak of synaptophysin described by Clift-O'Grady et al. (1990) in PC12 cells. This peak, which has been shown to contain endocytic organelles with the same physical properties as neuronal SVs (Clift-O'Grady et al., 1990), was not observed when extracts of transfected cells expressing synaptophysin as a foreign protein were analyzed by the same centrifugation conditions (our present results and Clift-O'Grady et al., 1990). Consequently, it appears that a distinct population of organelles with physical properties of authentic SVs may be formed only in cells that endogenously express synaptophysin together with other SV proteins.

The second peak of synaptophysin and other SV proteins identified in endocrine cells coincided with the single peak of transferrin receptors. This finding is consistent with the overlapping distribution of synaptophysin and transferrin receptor observed by immunofluorescence, equilibrium density centrifugation and immunoisolation and is in contrast with results reported by Clift-O'Grady et al. (1990). Using identical sedimentation conditions, Clift-O'Grady et al. did not report a codistribution between synaptophysin and internalized transferrin in PC12 cells. They concluded that synaptophysin and transferrin receptors were in different compartments and that the bulk of synaptophysin was present in an organelle that excludes receptor mediated endocytic markers. In distinction, our data demonstrate that a significant fraction of synaptophysin and other SV proteins are in the same organelles which contain a receptor mediated endocytic marker.

The compartments involved in the recycling of plasmalemmal receptors are thought to be represented by small vesicles and tubular-vesicular elements (early endosomes) that are dynamically (Yamashiro et al., 1984; Mellman et al., 1987) or possibly even physically interconnected (Hopkins et al., 1990). Early endosomes (Schmid et al., 1988), also referred to as receptosomes (Pastan and Willingham, 1985), sorting endosomes (Salzman and Maxfield, 1989), or CURL (Geuze et al., 1984) play a key role in sorting proteins internalized from the plasma membrane. It has been proposed that proteins destined for degradation remain in the vacuolar body of the early endosome, whereas proteins destined to recycle are concentrated in tubular extensions, which pinch off forming vesicles that recycle back to the plasma membrane (Geuze et al., 1984; Mellman et al., 1987). We hypothesize that synaptophysin and transferrin receptors are colocalized throughout the early recycling compartments of the endocytic pathway in CHO38 cells and in endocrine cells. However, in endocrine cells, synaptophysin together with other SV proteins, bud away from early endosomes to form a vesicle population separate from the vesicles that recycle plasmalemmal receptors. As suggested by our studies in pancreatic β -cells, these vesicles may have a specialized role in the secretion of nonpeptide molecules (Reetz et al., 1991). It will be of interest to determine whether the coexpression of other SVs membrane polypeptides in CHO38 cells results in a partial segregation of synaptophysin from transferrin receptors, a process analogous to that observed

in PC12 cells and RINm5F cells. It may be appropriate to restrict the use of the term SLMVs, previously applied by us to the bulk of the synaptophysin-positive vesicles of endocrine cells, to these vesicles devoid of transferrin receptors.

We have not detected transferrin receptors in SVs of adult neurons, consistent with the specialized function of these organelles (our unpublished observations). Thus, neurons like endocrine cells appear to have the biochemical machinery necessary to segregate efficiently synaptophysin from transferrin receptors. To determine whether a pool of synaptophysin overlapped with transferrin receptors in neurons, we have compared the distribution of synaptophysin and transferrin receptors in primary cultures of hippocampal neurons. In stage 4 neurons (Dotti et al., 1988), i.e., in neurons that have already established axonal and dendritic polarity, transferrin receptor immunoreactivity could be observed only in the cell soma and in dendrites. At this stage, synaptophysin immunoreactivity was concentrated preferentially in axons (this study and Fletcher et al., 1991), but it was also present in dendrites where it overlapped significantly with transferrin receptors at the level of resolution used. Preliminary immunofluorescence analysis of neurons before the establishment of cell polarity suggested an overlap between the distribution of transferrin receptors and synaptophysin throughout all cell processes analogous to the overlap between the distribution of the two proteins observed in endocrine cells by the same technique (our unpublished observations). These findings are consistent with the possibility that the interrelationships between the traffic of transferrin receptors and SV proteins observed in endocrine cells may also be seen in immature neurons in culture. We have previously suggested that SVs represent the neuron-specific adaptation of SLMVs. The present study raises the possibility that the evolution of SVs from SLMVs may be recapitulated in developing neurons in culture. It also suggests that the segregation of synaptophysin from transferrin receptors may take place in endosomes as well as at the level of the *trans*-Golgi network in polarized neurons.

The analysis of viral protein targeting in neurons has indicated that some basic mechanisms of protein sorting to different plasmalemmal domains may be similar in epithelial cells and in neurons (Dotti and Simons, 1990). Furthermore, these studies have suggested that the axonal membrane may be considered analogous to the apical plasma membrane and the dendritic membrane to the basolateral plasma membrane (Dotti and Simons, 1990). Our observation that the transferrin receptor, a basolateral recycling protein in epithelial cells (Fuller and Simons, 1986), is restricted to the dendrites of polarized primary neurons supports this hypothesis. Our data also support the idea that neurons have distinct axonal and dendritic recycling pathways analogous to apical and basolateral endocytic pathways found in epithelial cells (Simons and Wandinger-Ness, 1990).

The demonstration that the recycling pathway of synaptophysin partially overlaps with the receptor-mediated endocytic pathway is consistent with the observation that reformation of synaptic vesicles after intense nerve terminal stimulation appears to involve endosomal intermediates (Heuser and Reese, 1973; Valtorta et al., 1990). It is of interest that the *shibire* mutation of *Drosophila*, which has been shown to impair SV recycling (Koening and Ikeda, 1989; Roth, T. F., B. D. Holst, H. Kadhakrishna, and J. E.

Heuser. 1990. *J. Cell Biol.* 111:261a), also produces a general impairment of receptor-mediated endocytosis in all cells. It is well established that all cells are able to regulate the composition of their plasma membrane of continually inserting and removing selective components by a process of membrane recycling. In many cases, adaptations of recycling mechanisms have evolved for specialized cell functions. Some examples include the regulated insertion of functionally important proteins in the plasmalemma of specialized cells (e.g., proton pumps in intercalated cells of the kidney collecting duct and water channels in principal cells of the kidney collecting duct [Brown, 1989]; glucose transporters in adipocytes [Baly and Horuk, 1988; Suzuki and Kono, 1980]). The recycling pathways of SLMVs and SVs may also be considered cell specific adaptations in endocrine cells and neurons of a prototypic recycling pathway.

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