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Synaptophysin is targeted to similar microvesicles in CHO and PC12 cells

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Synaptophysin, an integral membrane protein of small synaptic vesicles, was expressed by transfection in fibroblastic CHO-K1 cells. The properties and localization of synaptophysin were compared between transfected CHO-K1 cells and native neuroendocrine PC12 cells. Both cell types similarly glycosylate synaptophysin and sort it into indistinguishable microvesicles. These become labeled by endocytic markers and are primarily concentrated below the plasmalemma and at the area of the Golgi complex and the centrosomes. A small pool of synaptophysin is transiently found on the plasma membrane. In CHO-K1 cells synaptophysin co-localizes with transferrin that has been internalized by receptor-mediated endocytosis. These findings suggest that synaptophysin in transfected CHO-K1 cells and neuroendocrine PC12 cells is directed into a pathway of recycling microvesicles which, in CHO cells, is shown to coincide with that of the transferrin receptor. They further indicate that fibroblasts have the ability to sort a synaptic vesicle membrane protein. Our results suggest a pathway for the evolution of small synaptic vesicles from a constitutively recycling organelle which is normally present in all cells.

Key words: endosomes/receptor mediated endocytosis/secretion/synaptic vesicles/transferrin receptor

Introduction

Nerve endings are densely populated by specialized secretory organelles, small synaptic vesicles, which are involved in the uptake and storage of non-peptide neurotransmitters and in their quantal secretion by exocytosis. The protein components of small synaptic vesicle membranes are currently the object of intense study and their structures and functions are beginning to be elucidated (reviewed in De Camilli and Navone, 1987; Trimble and Scheller, 1988). Those proteins that have been characterized appear to be concentrated on the membranes of all small synaptic vesicles, but largely excluded from the membranes of large dense core vesicles, i.e. the secretory organelles of neurons that release peptide neurotransmitters (Navone *et al.*, 1984, 1986). Endocrine cells also express several synaptic vesicle membrane proteins in a microvesicular compartment of unknown function (Navone *et al.*, 1986). These small vesicles are distinct from the dense core secretory vesicles in endocrine cells, although some reports find a small percentage of the synaptic vesicle proteins in the latter (Lowe *et al.*, 1988).

Many aspects of the life cycle of small synaptic vesicles are well described morphologically. However, the molecular mechanisms involved in determining their biogenesis and in regulating their intracellular traffic are still poorly understood (Tartakoff, 1987; Kelly, 1988). Membrane proteins of small synaptic vesicles have been detected only in neurons and endocrine cells. Are the small synaptic vesicles in neurons related to vesicles that are common to all eukaryotic cells? If so, can one learn more about the properties of small synaptic vesicles by comparing their biochemical composition and their intracellular traffic to those of related vesicles in less specialized cells? Finally, what is the nature of the endocrine microvesicles and how are they related to small synaptic vesicles and to vesicles common to all eukaryotic cells?

To explore the possible relation of the neuronal and endocrine pathways to a vesicular pathway present in all cells, we have expressed by transfection in CHO-K1 cells a relatively abundant protein of small synaptic vesicles. The goal of these studies was to determine how such a protein was sorted in a cell that does not normally express it. If a compartment related to small synaptic vesicles, and to their endocrine counterpart, exists in all cells, then the protein might be targeted to such a compartment and could be a tool in studying it.

As a model protein we selected synaptophysin, a well characterized intrinsic membrane protein of synaptic vesicles (Jahn *et al.*, 1985; Wiedenmann and Franke, 1985; Navone *et al.*, 1986). Synaptophysin is a glycosylated integral membrane protein of M_r38 000. It has four transmembrane spanning regions and a cytoplasmic domain that contains ten copies of a tyrosine-rich repeat (Südhof *et al.*, 1987b, c; Buckley *et al.*, 1987; Leube *et al.*, 1987; Johnston *et al.*, 1989). We now show that when expressed in CHO cells, synaptophysin is full processed. It is efficiently targeted to a distinct microvesicular compartment that is labeled by endocytic markers and appears to recycle via the plasma membrane. When transfected CHO cells are compared to neuroendocrine PC12 cells that express the protein naturally, synaptophysin behaves similarly in both cells. Our findings characterize the synaptophysin-containing microvesicles of endocrine cells as a constitutively recycling organelle which can be found in CHO cells after transfection of synaptophysin. These results suggest a pathway for the evolution of synaptic vesicles from a constitutively recycling microvesicular organelle of eukaryotic cells.

Results

A rat synaptophysin expression vector was constructed that induces the transcription of a full length synaptophysin

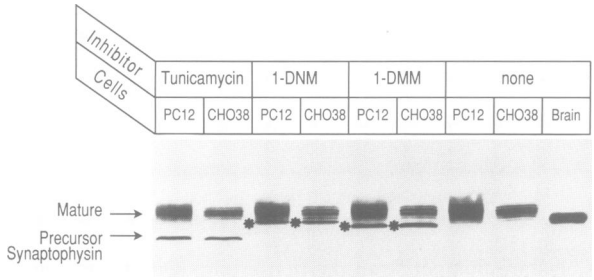

Fig. 1. Glycosylation of synaptophysin in PC12 and transfected CHO cells. Cells were treated as described in Materials and methods with tunicamycin, 1-deoxynojirimycin (1-DNM) or 1-deoxymannojirimycin (1-DMM). Total cell protein (125 μg) was separated by gel electrophoresis, immunoblotted, and probed with an anti-synaptophysin monoclonal antibody as described (Johnston *et al.*, 1989). The glycosylation intermediates of synaptophysin after 1-DNM and 1-DMM treatment are only slightly smaller than the fully processed form and are labeled by asterisks. Rat brain homogenate is electrophoresed on the right to demonstrate the slightly smaller size of glycosylated synaptophysin in the central nervous system.

message under the control of the cytomegalovirus promoter. Permanent CHO cell lines expressing synaptophysin were established after co-transfection of the plasmid with a neomycin resistance gene. When compared to untransfected CHO cells and CHO cells transfected with a neomycin resistance plasmid only, CHO cells expressing synaptophysin exhibited a somewhat slower growing rate, and under phase-contrast observation appeared slightly larger and more flattened on the dish.

When analyzed by immunoblotting, these cells were found to express synaptophysin in a heterogeneously glycosylated form indistinguishable from that of rat brain synaptic vesicles (Figure 1). It has been previously noted that synaptophysin exhibits a differential form of glycosylation in central neurons and peripheral endocrine cells (Navone *et al.*, 1986). The glycosylation reactions of synaptophysin in transfected CHO cells and in PC12 cells were probed by treating the cells in culture with drugs that specifically inhibit *N*-linked glycosylation at different steps (Fuhrmann *et al.*, 1984). The treated and untreated cells were then analyzed by immuno-

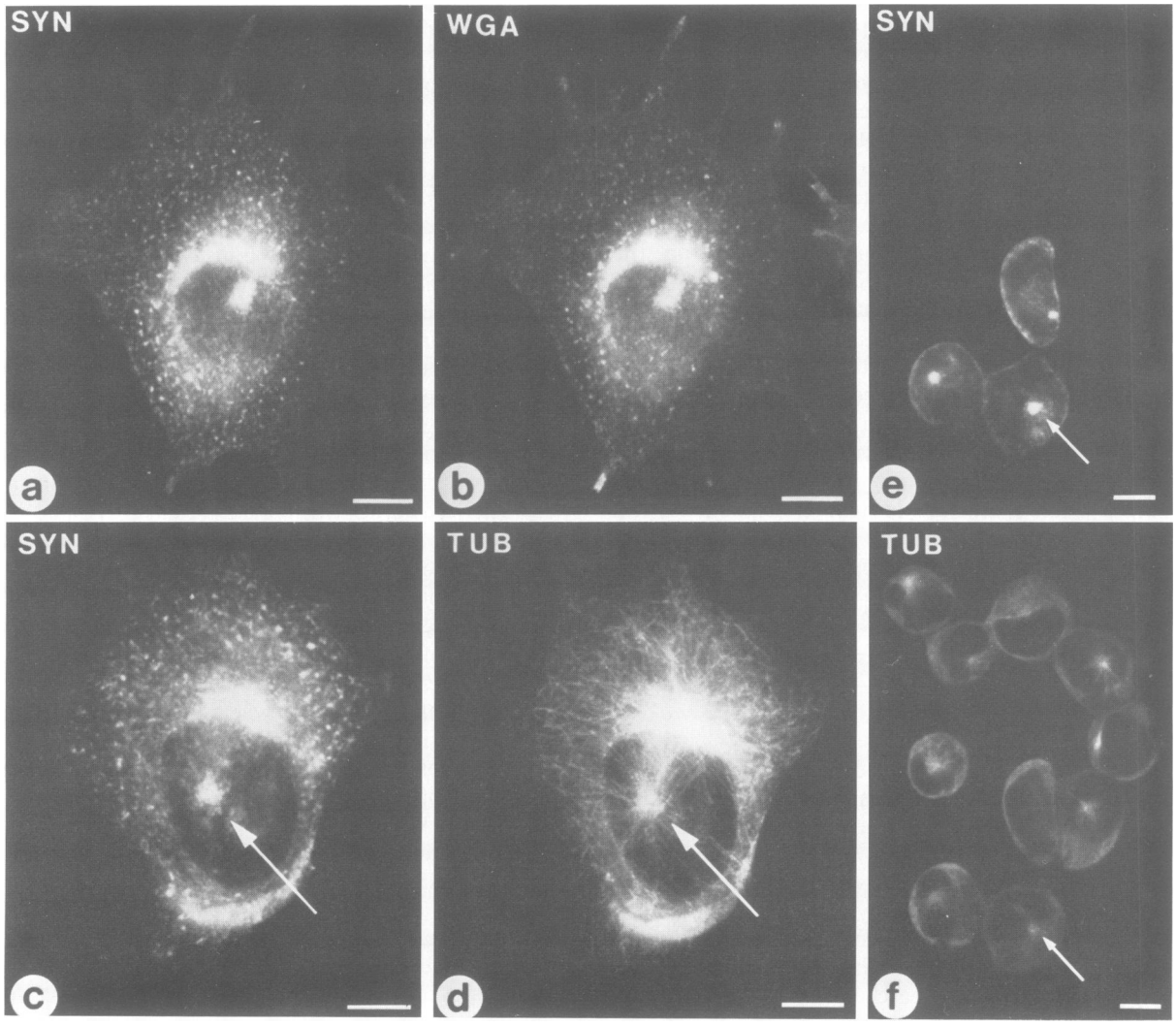

Fig. 2. Immunofluorescence localization of synaptophysin in transfected CHO cells. Three pairs of pictures are shown that depict the same cells double-labeled with antibodies directed against synaptophysin (**a,c,e**) and with either wheat germ agglutinin (**b**) or with antibodies against β-tubulin (**d,f**). The fluorophors used were rhodamine (a,c,e) or fluorescein (b,d,f). The perinuclear region which is brightly stained by synaptophysin antibodies corresponds to the Golgi complex area as demonstrated by the similar perinuclear wheat germ agglutinin stain while the punctate staining of the surrounding cytoplasm is different in the two fields (a,b). A prominent accumulation of synaptophysin immunoreactivity can be seen at microtubular organizing centers (arrows in c,d). The double-labeling shown in (e) and (f) is from a heterogeneous culture of synaptophysin-expressing and non-expressing cells, to demonstrate the specificity of the synaptophysin immunostain. (Calibration bars 10 μm.)

blotting. In both cell types synaptophysin antibodies labeled on immunoblots a specific protein band of M_r40 000 that was not observed in transfected control CHO cells expressing the neomycin resistance marker only. Tunicamycin treatment of the cells resulted in the appearance of a new M_r34 000 band that presumably represents the primary translation product (left lanes, Figure 1). New immunoreactive bands representing processing intermediates appeared as a function of the glycosylation inhibitors 1-deoxynojirimycin and 1-deoxymannojirimycin (asterisks in Figure 1). Probing the glycosylation reactions by immunoblotting reveals fully glycosylated forms of synaptophysin in addition to the intermediates produced after glycosylation inhibition because of the long half-life of synaptophysin in transfected CHO cells and in PC12 cells. This was found to exceed two days in experiments using immunoprecipitation after pulse-chase-labeling of the cells with [^{35}S]methionine (data not shown). Glycosylated synaptophysin from PC12 cells did not electrophorese sharply. However, the apparent sizes of the primary translation products and of the various glycosylation intermediates were identical in PC12 and CHO cells, and the relative intensities of the bands were very similar. These results suggest that the transfected CHO cells process synaptophysin in an identical manner and at a similar rate as do PC12 cells, and that they express comparable amounts of the protein.

Analysis of the intracellular localization of synaptophysin in transfected CHO cells by immunofluorescence revealed a punctate distribution of immunoreactivity. In Figure 2, three pairs of pictures show the same microscopic fields double-labeled either for synaptophysin and wheat germ agglutinin binding sites (2a and b), or for synaptophysin and β-tubulin (2c and d; e and f). Very similar results were obtained in parallel experiments with PC12 cells (data not shown). Puncta of synaptophysin immunoreactivity were scattered throughout the cytoplasm but were present at higher concentrations at microtubule organizing centers where the Golgi complex is localized. This was demonstrated by comparison with the distribution of β-tubulin (arrows in Figure 2c and d) and of binding sites for wheat germ agglutinin (Figure 2a and b), a cytochemical marker for the Golgi complex (Tartakoff *et al.*, 1983). A less significant accumulation of immunoreactivity was sometimes visible at the cell periphery, in particular on rounded cells (Figure 2e). The same pattern of immunoreactivity was observed with CHO cells from six independent transfections and in transient expression experiments using CHO and COS cells. No staining was observed in transfected or untransfected control cells. Similar results were obtained using monoclonal or polyclonal antibodies directed against synaptophysin, but not using a panel of control antibodies or antibodies directed against other proteins of small synaptic vesicle membranes. As a further control, cell populations containing both synaptophysin-expressing and non-expressing cells were double-labeled for synaptophysin and β-tubulin (Figure 2e and f). Clearly the intracellular staining is highly specific for synaptophysin.

Immunogold labeling of ultrathin frozen sections was performed to analyze the subcellular localization of synaptophysin at the electron microscopic level in transfected CHO cells (Figures 3 and 4). This was compared to that observed in native PC12 cells (Figure 5). In order to probe the dynamics of the traffic of synaptophysin-rich membranes these cells were studied both without treatment (Figure 3) and after preincubation for 1 h in extracellular horseradish peroxidase as a marker for endocytic pathways (Figures 4 and 5).

In CHO cells expressing synaptophysin, heavy gold labeling was observed on small vesicular structures (Figures 3 and 4), while non-expressing control cells showed no specific label (Figure 4g). The intracellular distribution of the microvesicles exhibited a consistent pattern. They tended to occur in small clusters that were primarily observed in the cortical regions of the cell (Figure 4b) or at the *trans*-side of the Golgi complex (Figure 3a). When a centriole was seen it was always surrounded by a 'cloud' of labeled microvesicles (not shown). In general, little or no labeling of the plasmalemma was observed (Figures 3a,b,d and 4a–c), but a few membrane stretches with relatively high numbers of gold particles could be found (Figure 4d).

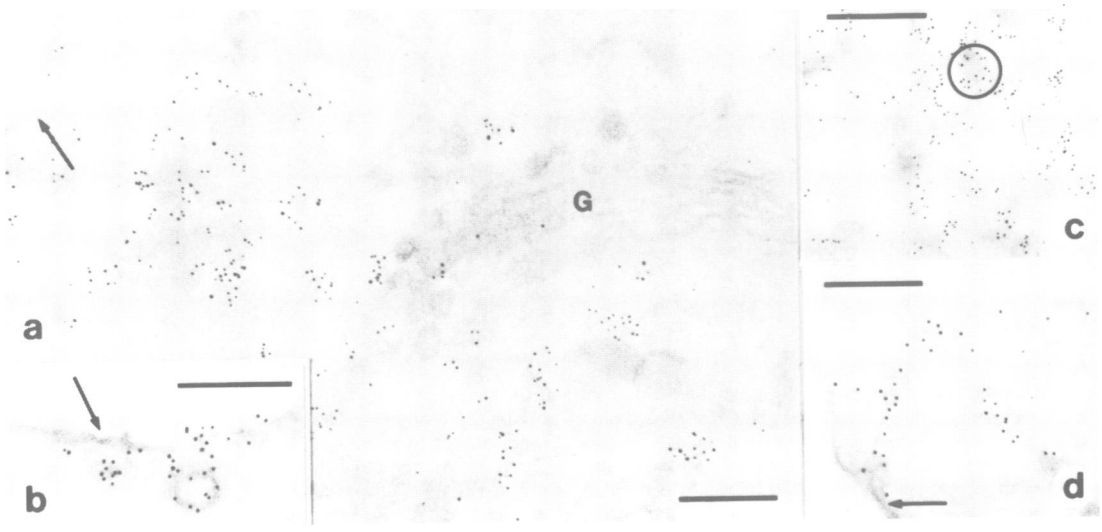

Fig. 3. Immunogold localization of synaptophysin in ultrathin frozen sections of transfected CHO cells. (**a**) and (**c**) show that synaptophysin is concentrated in microvesicles, including microvesicles localized in proximity to the Golgi complex (G). The cisternae of the Golgi, however, are unlabeled. A labeled microvesicle fused with the plasmalemma (omega profile) and an invaginated labeled tubule are visible in (**b**) and (**d**). Scattered gold particles are visible on the plasmalemma which is marked by arrows. (Calibration bars 200 nm.)

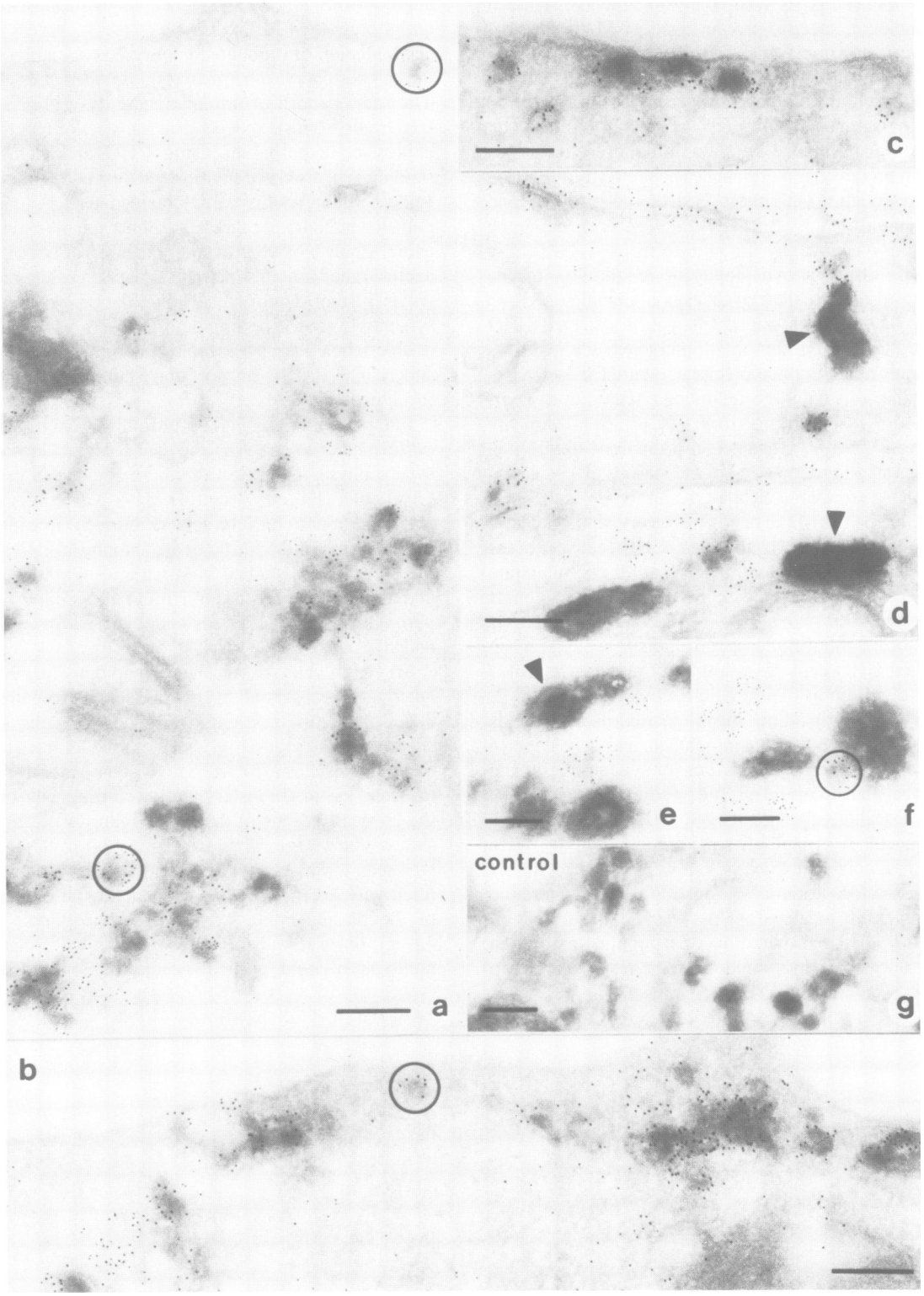

Fig. 4. Immunogold localization of synaptophysin in ultrathin frozen sections of transfected CHO cells that were exposed to horseradish peroxidase to label endocytic compartments. The various fields show that synaptophysin is primarily concentrated on synaptic vesicles (circles), most of which are filled with peroxidase reaction product. Larger organelles which contain peroxidase reaction product are synaptophysin-negative (arrowheads in **d**,**e**). In (**f**), a gold labeled vesicle closely adjacent to one of those organelles is circled. In general no labeling is visible on the plasmalemma (**a**,**b**,**c**), but occasionally stretches of labeled plasmalemma can be seen (d). In (**g**), a transfected CHO cell that does not express synaptophysin is shown as a control. In this field, peroxidase-containing microvesicles are unlabeled by gold particles. (Calibration bars 200 nm.)

Sometimes labeled vesicles (omega figures) or invaginated tubules were observed that were continuous with the plasmalemma (Figure 3b and d).

No significant immunostaining was found on the cisternae of the Golgi complex (Figure 3a) or on the rough endoplasmic reticulum (not shown). Synaptophysin has four

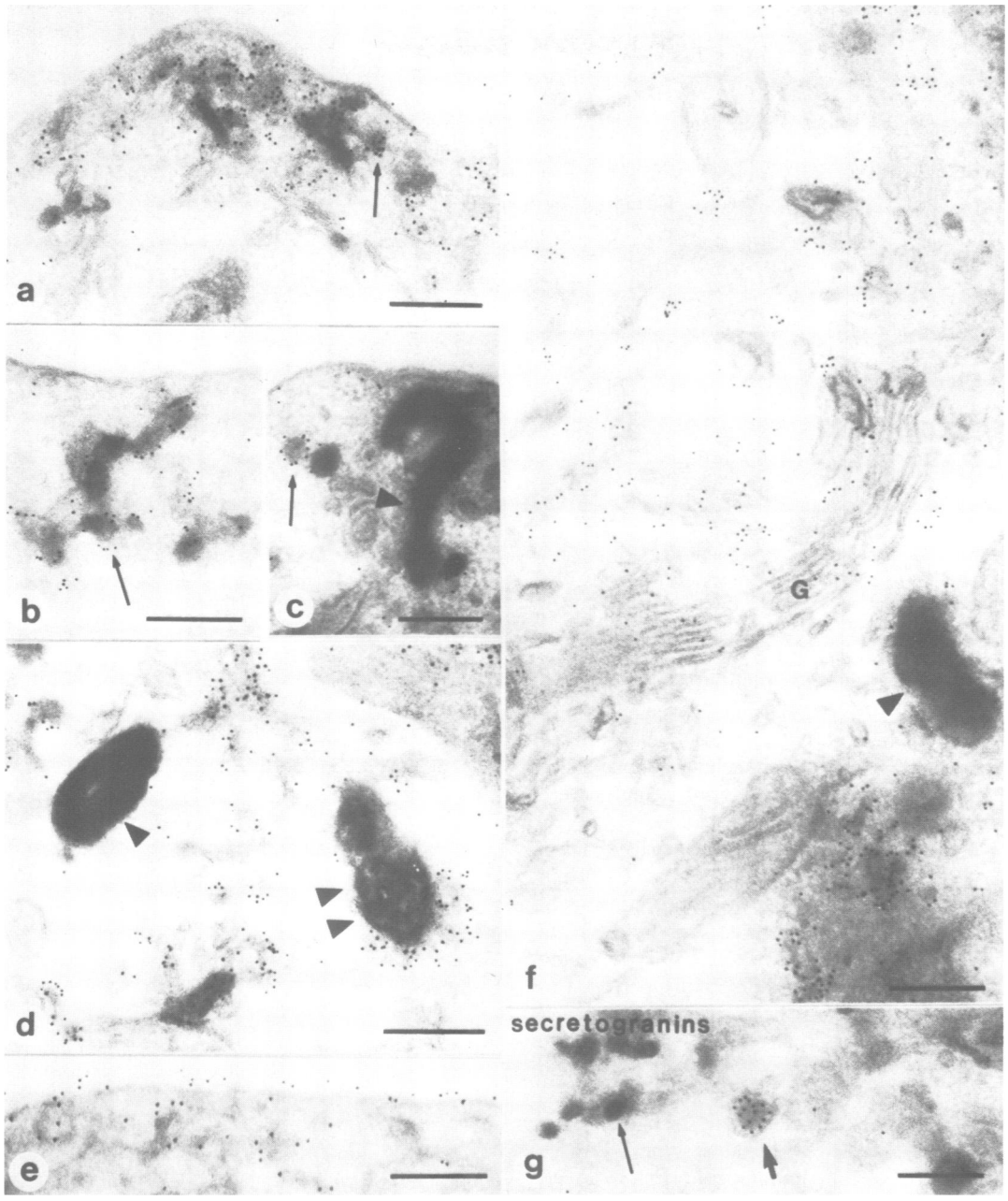

Fig. 5. Immunogold labeling of synaptophysin (a–f) and of secretogranin (**g**) in ultrathin frozen sections of undifferentiated PC12 cells that were exposed to horseradish peroxidase to label endocytic compartments. (**a–d**) show that synaptophysin is concentrated on small vesicular profiles often labeled (arrows) by peroxidase reaction product. The membranes of larger peroxidase-positive vesicles are not labeled by immunogold (arrowheads in c and d), but immunogold-decorated vesicles can be seen in close association. One of those organelles surrounded by a 'cloud' of positive vesicles is visible in (d) (double arrowheads). Note lack of labeling of the plasmalemma in (a–c), and a stretch of labeled plasmalemma in (d). Field (**f**) shows that intense immunolabeling can be seen in the proximity of the Golgi complex, but that no significant labeling is visible on the Golgi cisternae. (**g**) demonstrates immunogold labeling for secretogranins. Peroxidase-containing microvesicles are unlabeled by gold particles. These are concentrated on the core of a secretory granule. (Calibration bars 200 nm.)

transmembrane regions and is glycosylated on intravesicular sequences. Since the polyclonal and monoclonal antibodies used in this study are directed aginst the cytoplasmic tail of synaptophysin (Johnston *et al.*, 1989), lack of labeling of the endoplasmic reticulum and of the Golgi cisternae is most likely due to the low rate of synthesis of the molecule and not to a glycosylation-dependent change in its antigenic properties in the Golgi complex.

The subcellular distribution of synaptophysin in transfected CHO cells was indistinguishable from that of synaptophysin in undifferentiated PC12 cells (Figure 5). Its pattern also duplicated the distribution of the protein previously described in chromaffin and pituitary cells (Navone *et al.*, 1986). The predominant immunolabeling of microvesicular structures (Figure 5a–d,f), the lack of labeling on cisternae of the Golgi complex (Figure 5f) and on the endoplasmic reticulum (not shown), and the clusters of labeled microvesicles at the cell periphery (Figure 5a) were also observed in PC12 cells. Little labeling of the plasma membrane was generally observed in PC12 cells, but again there were occasional

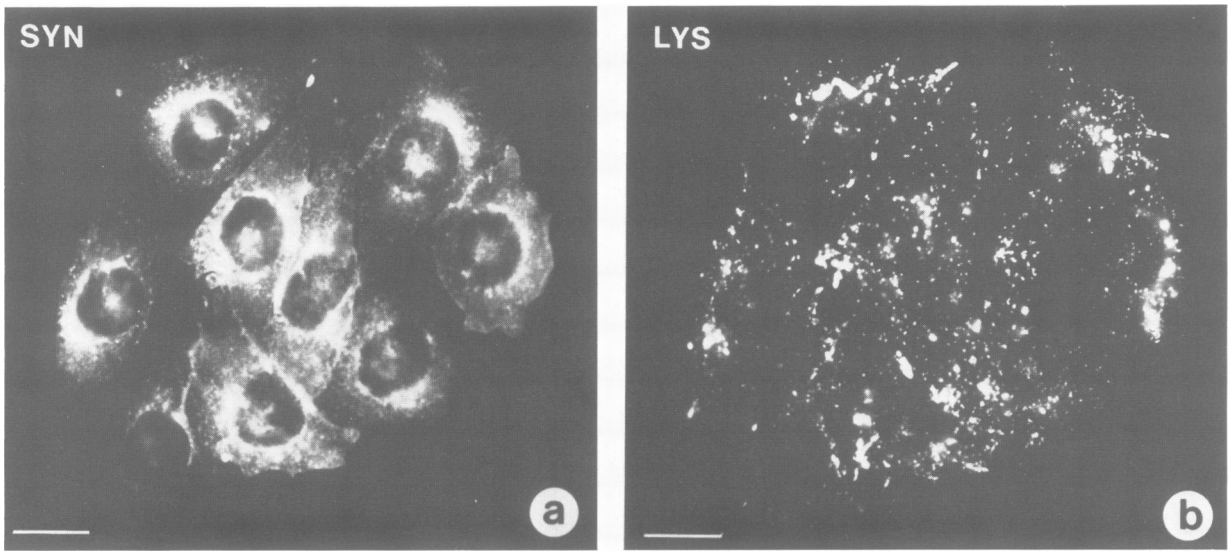

Fig. 6. Localization of synaptophysin (**a**) and of the lysosomal membrane marker protein 1gp96 (**b**) in transfected CHO cells by double-labeling immunofluorescence. In (b) large, bright dots represent lysosomes which can be seen to have an intracellular distribution very different from that of synaptophysin immunoreactivity visible in (a). Note the characteristic pattern of synaptophysin immunoreactivity represented by very fine puncta dispersed throughout the cytoplasm and concentrated at microtubule-organizing centers. (Calibration bars 20 μm.)

labeled membrane segments (cf. Figure 5a and e). The specificity of the labeling was demonstrated by staining PC12 cells for secretogranins I and II, two secretory proteins stored in granules (Rosa *et al.*, 1985). After such immunolabeling, only the cores of large-dense core secretory granules were stained, but not the microvesicles (Figure 5g).

The presence of synaptophysin at the cell surface and in invaginated 'omega' figures suggested that in both CHO and PC12 cells synaptophysin-containing membranes fuse with the plasmalemma. This hypothesis was supported by the finding that after a 1 h incubation of both cell types in the presence of the extracellular tracer horseradish peroxidase, a large fraction (but not all) of the microvesicles labeled by synaptophysin−immunogold also contained peroxidase (Figures 4 and 5). On the other hand, not all of the peroxidase-positive microvesicles were labeled by gold. The membranes of the larger vacuoles that were strongly positive for peroxidase and most likely represent lysosomes and large endosomes were not labeled by immunogold (Figure 4d−f; Figure 5c and d). However, synaptophysin-positive tubules and vesicles could be seen, in some cases, in close proximity to such vacuoles (Figure 4e and f). These images are consistent with the possibility that some of these vesicles might represent synaptophysin-rich tubular extensions continuous with the vesicular portion of large endosomes (Marsh *et al.*, 1986; Geuze *et al.*, 1987). On the whole, synaptophysin containing microvesicles are very similar in properties and localization in transfected CHO and native PC12 cells.

The conclusion that synaptophysin was not present at a significant concentration in the membranes of lysosomes was supported by double immunofluorescence experiments in both CHO and PC12 cells. In one set of experiments cells were double-labeled for synaptophysin and for a marker of lysosomes, the lysosomal membrane protein Igp96 (Figure 6; S.Schmid, R.Fuchs, H.Plutner and L.Mellman, personal communication). In the other set of experiments cells were labeled for synaptophysin and for DAMP, an immunocytochemical marker for acidic intracellular compartments

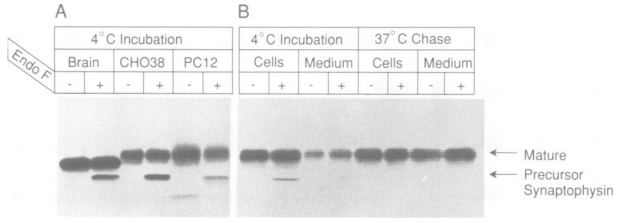

Fig. 7. Synaptophysin is partially susceptible to endoglycosidase F cleavage in intact cells. In **A**, synaptophysin-expressing CHO cells, native PC12 cells and rat brain homogenates were incubated at 4°C in the absence or presence of endoglycosidase F in PBS. In **B**, synaptophysin-expressing CHO cells were first incubated with or without endoglycosidase F at 4°C, then washed and further incubated for 30 min at 37°C in the absence of endoglycosidase F. Media and washes after each incubation were saved, pooled and cellular debris was concentrated by centrifugation. This was analyzed in parallel with the cells after each incubation step by SDS−PAGE and immunoblotting. 125 μg cell protein were loaded per lane, media and washes loaded corresponded to ~10× as many cells.

(Anderson *et al.*, 1984; data not shown). At the light microscopic level of resolution it labels primarily large acidic compartments such as lysosomes and large endosomes. Synaptophysin immunoreactivity was found to have a very different distribution from both lysosomal and DAMP immunoreactivities.

The results of immunoelectron microscopy suggested that at any given time, both in PC12 and CHO cells, most of the synaptophysin was intracellular but that a small percentage was localized in the plasmalemma. The possibility that synaptophysin recycles via the plasma membrane was further suggested by the presence of extracellular tracer in synaptophysin-positive microvesicles. To confirm by an independent approach that synaptophysin is partially surface exposed we first unsuccessfully tried to probe intact cells with antibodies raised against the intravesicular loops of synaptophysin (Johnston *et al.*, 1989). However, while these antibodies reacted well with synaptophysin on immunoblots,

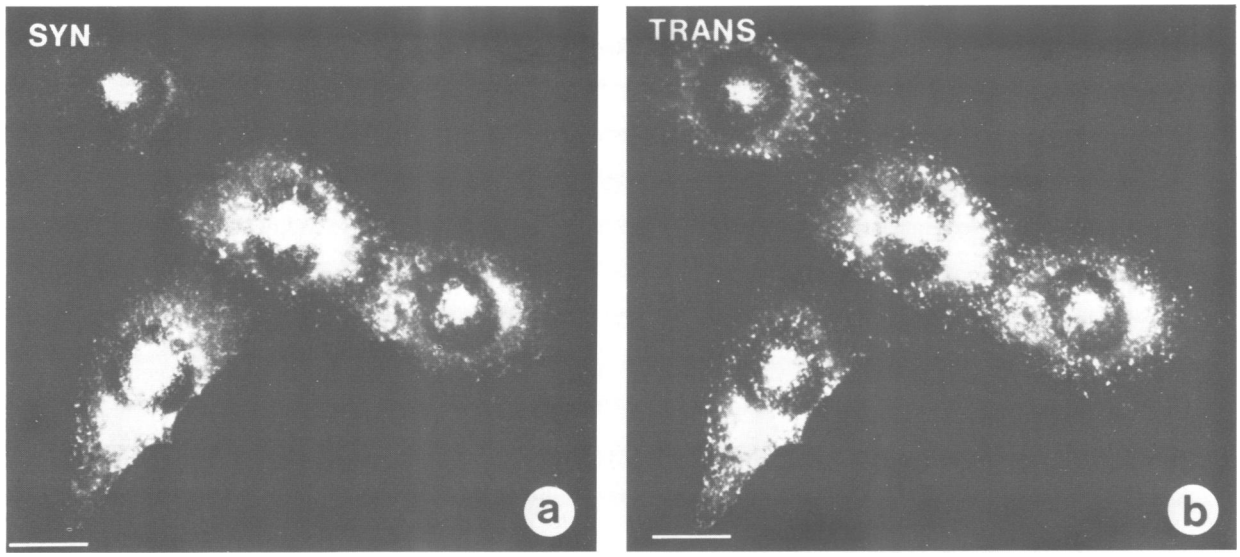

Fig. 8. Localization of synaptophysin (**a**) and of internalized transferrin (**b**) in transfected CHO cells by double-label immunofluorescence. Cells were exposed to 10–20 μg/ml human transferrin for 30 min as described in Materials and methods. The two patterns of immunoreactivity are strikingly similar. The cell at the upper left corner expresses lower levels of synaptophysin than the other three cells in the field. (Calibration bars 15 μm.)

none of them reacted with the protein in immunocytochemical experiments. We therefore tested if a fraction of the synaptophysin in intact CHO and PC12 cells was susceptible to digestion by endoglycosidase F which cleaves *N*-linked sugars at their attachment sites (Elder and Alexander, 1982). Endoglycosidase F was added to the cells precooled on ice in order to prevent its endocytosis. Before harvesting, the cells were washed numerous times. After endoglycosidase F treatment a small percentage of synaptophysin in PC12 and in transfected CHO cells was converted to a smaller form that was the same size as the primary translation product (Figure 7A). The observed cleavage by endoglycosidase F was not due to digestion of lysed dead cells on the dishes. This was shown in experiments where the cells were washed before harvesting and the washed off material was analyzed and found to contain very little cleaved synaptophysin (Figure 7B). Trypan blue exclusion tests were performed and demonstrated that the attached cells were fully viable. When the cells were warmed to 37°C after endoglycosidase F treatment they quickly lost the endoglycosidase F cleavage product (Figure 7B). Again the washes were analyzed and found not to contain the cleaved product, confirming that endoglycosidase F-cleaved synaptophysin was not selectively localized to a small percentage of lysed cells. The rapid clearance of endoglycosidase F-cleaved product may be due to its preferential degradation.

Taken together, these findings suggested that synaptophysin is targeted to a cellular pathway which participates in endocytosis, but which segregates from large endosomes and lysosomes in the cells. One such vesicular pathway which is expressed in a large variety of cells is the pathway which recycles transferrin receptors and possibly other receptors. To determine the relationship between microvesicles to which synaptophysin is targeted in CHO cells and the microvesicles which recycle transferrin, another set of double-labeling experiments was performed. CHO cells were first incubated for 1 h in the presence of 10–20 μg/ml human transferrin, i.e. a concentration sufficient for binding transferrin to a large majority of intracellular transferrin receptors via a receptor-mediated uptake, but insufficient to produce a significant bulk uptake of the molecule. Subsequently these cells were double-stained by immunofluorescence for synaptophysin and for transferrin. Results of these experiments indicated a striking coincidence between the puncta of synaptophysin immunoreactivity and transferrin-positive puncta as shown in Figure 8.

Discussion

We have expressed synaptophysin, a major intrinsic membrane protein of small synaptic vesicles, in a fibroblastic cell line. Morphological and biochemical methods were used to characterize its localization and properties in the transfected fibroblasts and in neuroendocrine PC12 cells that naturally express the protein.

Our results suggest that synaptophysin is glycosylated similarly in both cell types and is targeted to a similar population of pleomorphic microvesicles. These microvesicles are scattered throughout the cytoplasm but are present at a particularly high concentration in the Golgi–centrosomal area, and often occur in clusters in the cortical regions of the cell under the plasmalemma. The microvesicles sequester horseradish peroxidase when cells are exposed to it as an endocytic marker, suggesting that they represent a compartment in functional continuity with the plasma membrane. While little synaptophysin was observed in the plasmalemma, synaptophysin-rich membrane patches and 'omega' figures at the plasma membrane were found and partial surface exposure of synaptophysin was also demonstrated biochemically in both cell types.

Synaptophysin was not found on the vesicular portions of the large vacuoles (probably large endosomes and lysosomes) that were highly enriched in horseradish peroxidase used as an endocytic marker. However, synaptophysin-positive small membranous profiles were often visible in close proximity and sometimes directly adjacent to some of these large vacuoles. These structures may represent tubulo-vesicular extensions of early endosomes (Marsh *et al.*, 1986). It

is thought that such tubulo-vesicular extensions have a composition distinct from the vesicular portion of the endosomes and that they are enriched in proteins destined for recycling to the plasmalemma (Marsh *et al.*, 1986; Geuze *et al.*, 1987). Together these findings are consistent with the possibility that synaptophysin follows the pathway of recycling cell-surface molecules. The co-localization of synaptophysin and of internalized transferrin observed in our double-immunofluorescence experiments in CHO-K1 cells strongly support this possibility. It is of interest that in CHO cells the transferrin and LDL receptors have previously been shown to recycle via a pleomorphic microvesicular population similar to those described here (Yamashiro *et al.*, 1984; Goldstein *et al.*, 1985).

Cisternae of the Golgi complex and of the rough endoplasmic reticulum were not labeled at significant levels by synaptophysin antibodies in transfected CHO and PC12 cells, suggesting that at any given moment only very low levels of synaptophysin are being synthesized. This observation, together with the lack of labeling on lysosomes suggested by the peroxidase uptake experiments and by the double-immunofluorescence experiments, is in agreement with the relatively long half-life of the molecule.

The similarity between the localization of synaptophysin in CHO cells (cells which naturally do not express the protein) and in PC12 cells (cells which naturally express the protein) indicates that the biochemical machinery which is involved in sorting the protein is not restricted to endocrine and neuronal cells and sheds light on the cell biology of these organelles.

What is the nature of the synaptophysin-positive microvesicles in endocrine cells and in transfected CHO cells? At least two major possibilities can be considered: one is that the expression of synaptophysin induces the formation of a new population of microvesicles, the other is that synaptophysin is targeted to a pre-existing vesicular pathway. The similarity of synaptophysin-containing microvesicles to microvesicles which recycle transferrin (via its binding to the transferrin receptor) virtually rules out the first possibility. In addition, we have observed that the distribution of internalized transferrin, as assessed by immunofluorescence, is similar in CHO cells which express and which do not express synaptophysin. This speaks against the possibility that expression of synaptophysin diverts a significant portion of transferrin receptors to a newly created intracellular organelle, and further suggests that synaptophysin does not greatly modify the pathway to which it is targeted. However, an amplification of such a pathway analogous to the amplification of the endoplasmic reticulum induced by the overexpression of HMG-CoA reductase (Anderson *et al.*, 1983) cannot currently be excluded.

Our findings indicate that synaptophysin containing microvesicles of endocrine cells are related to the pathway involved in the recycling of receptors in eukaryotic cells. Small synaptic vesicles of neurons may represent a cell-specific adaptation of this pathway. While the synaptophysin containing microvesicles of endocrine cells are similar in morphology and in intracellular distribution to synaptophysin-containing microvesicles in CHO cells, small synaptic vesicles of neurons exhibit highly specific features. Both CHO and endocrine microvesicles are pleomorphic (round, oval, tubular), with a size range larger than that of small synaptic vesicles. In addition, in both cells the microvesicles are highly concentrated in the area of the Golgi complex and the centrosomes, suggesting that their recycling involves, at least to some extent, a microtubule mediated shuttling between the cell periphery and the *trans*-region of the Golgi complex. In contrast, small synaptic vesicles have a very homogeneous size in the 50 nm range and are localized primarily at the cell periphery, reflecting a predominance of local recycling at the cell periphery. This may represent a special adaptation of this pathway to its role in synaptic transmission.

The present study makes possible new approaches to explore how highly specialized small synaptic vesicles may be related to organelles operating in all cells. The ability to study an organelle related to synaptic vesicles in a cell type that does not normally express synaptic vesicle proteins allows genetic and biochemical experiments. These will attempt to investigate the physiological role of individual proteins of small synaptic vesicles and of specific domains of these proteins in sorting and recycling processes.

Materials and methods

Materials

Recombinant DNA and tissue culture materials were obtained from previously reported sources (Südhof *et al.*, 1985, 1987a). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and used after purification on 20% polyacrylamide cells. Glycoprotein processing inhibitors were purchased from Genzyme and endoglycosidase F from Boehringer Mannheim. CHO-K and COS cells were a gift of Dr J.L.Goldstein, UT Southwestern Medical School, and PC12 cells from Dr Paul Greengard, Rockefeller University and Dr Lloyd Greene, Columbia University. The different cytochemical reagents were from the following sources (suppliers or references in parentheses): polyclonal and monoclonal anti-synaptophysin antibodies (Jahn *et al.*, 1985; Johnston *et al.*, 1989); anti-DAMP antibodies (Anderson *et al.*, 1984); anti-secretogranin I and -secretogranin II (Dr W.Huttner, EMBL, FRG); monoclonal anti-1gp96 (Dr I.Mellman, New Haven, CT); polyclonal anti-transferrin (Accurate Chem. and Sci. Corp., NY); rhodamine and fluorescein-conjugated goat anti-rabbit and goat anti-mouse IgGs (Cappel-Organon Teknika and Sigma); gold-conjugated goat anti-rabbit IgGs (Janssen, Inc., Belgium); protein A−gold conjugates (Dr F.Navone, UCSF, USA), anti-β-tubulin antibodies (Amersham, Inc.) and fluorescein-conjugated wheat germ agglutinin (Vector Labs, Inc. and Molecular Probes, Inc.).

Plasmid construction

A full length cDNA encoding rat synaptophysin (pCMV38-1) was constructed by fusing the rat cDNA sequence (Südhof *et al.*, 1987b,c) 121−1259 nt to a synthetic sequence of nucleotides 19−120 as deduced from the rat synaptophysin gene. The full length construct was verified by sequencing and cloned into the *Kpn*I−*Hin*dIII sites of pCMV-1, a eukaryotic expression vector that uses the cytomegalovirus immediate early promoter to drive transcription (D.W.Russell, personal communication).

Cell culture and transfection

CHO-K1 and COS cells were maintained, transfected and selected as described (Wigler *et al.*, 1979; Südhof *et al.*, 1987a). PC12 cells were grown in 85% DMEM, 10% FBS, 5% horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Permanent lines expressing synaptophysin were obtained after co-transfecting pCMV38-1 with pSVNeo and selecting with 700 μg/ml G418. Cell lines were screened by immunoblotting and immunofluorescence and subcloned repeatedly. All data shown were obtained with a single subclone (5-3:13) but several independent subclones were found to have the same characteristics. Cells transfected only with the neomycin resistance marker and untransfected cells were used as controls.

Glycosylation inhibition

5×10^5 CHO and 1×10^6 PC12 cells were grown overnight in 35 mm culture wells, washed three times with phosphate-buffered saline pH 7.2 (PBS), and treated for 15 h at 37°C with either tunicamycin (20 μg/ml), 1-deoxynojirimycin (5 μM), or 1-deoxymannojirimycin (5 μM) in medium. Monolayers were washed with PBS and scraped in lysis buffer (10 mM Hepes pH 7.4, 0.2 M NaCl, 2.5 mM $MgCl_2$, 2 mM EDTA, 1%

PMSF, 5% NP-40). Samples (125 μg) were further solubilized in 2 × treatment buffer (0.125 M Tris pH 6.8m, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), boiled for 3 min and analyzed by 12% SDS–PAGE (Laemmli, 1970). Gels were elecrophoretically transferred to nitrocellulose and probed with polyclonal or monoclonal antibodies as described (Johnston *et al.*, 1989).

Endoglycosidase F experiments

1×10^6 CHO or PC12 cells were grown overnight in 100 mm dishes, washed three times in ice-cold PBS in the cold room and incubated for 6 h at 4°C with 200 U/ml endoglycosidase F in PBS pH 7.2. The incubation buffer was removed, monolayers were washed three times in ice-cold PBS and solubilized in lysis buffer. For chase experiments, warm medium was added to the cells after washing and they were incubated at 37°C for 30 min before harvesting. The media and washes from the 4°C endoglycosidase F incubation and the 37°C chase were saved and pooled separately. Their cellular debris was concentrated by centrifugation for 1 h at 150 000 *g* in a Beckman 70 Ti rotor. Samples (125 μg monolayer protein or the total cellular pellet of the pooled incubations and washes) were analyzed by 10% PAGE and immunoblotting as described (Johnston *et al.*, 1989).

Transferrin uptake

Cells were depleted of endogenous transferrin by subjecting them to washes and an incubation (60 min) with serum free medium at 37°C. Cells were then incubated for 30 min in serum free medium containing 10–20 μg/ml diferric (human) transferrin prepared according to the procedure of Bates (1973). At the end of the uptake period, cells were rinsed quickly with ice-cold medium without transferrin, then fixed and processed for immunofluorescence as detailed below.

Immunofluorescence staining

Mock or synaptophysin transfected CHO cells, grown on glass coverslips to 50–70% confluency, were fixed at 4°C with either (i) ice-cold 4% formaldehyde [freshly prepared from paraformaldehyde in 0.12 M Na-phosphate pH 7.4 (1 h)] or (ii) periodate-lysine-paraformaldehyde fixative (McLean and Nakane, 1974) (2 h). The second fixative was used for lysosome/synaptophysin double-labeling. The first fixative was used in all other cases. The cells were washed with 0.12 M Na-phosphate, permeabilized for 30 min in high salt PBS–BSA (20 mM Na-phosphate, 0.5 M NaCl, 3% BSA) supplemented with detergent (see below) and processed for single- or double-label immunofluorescence essentially as described (De Camilli *et al.*, 1986). The following antibody combinations were used for double-labeling: (i) polyclonal affinity-purified antibody directed against synaptophysin and a mouse monoclonal antibody (mAB E9A) directed against the lysosomal glycoprotein lgp96, (ii) polyclonal affinity-purified antibody directed against synaptophysin and a mouse monoclonal antibody directed against β-tubulin or (iii) polyclonal antibody directed against transferrin and a mouse monoclonal antibody directed against synaptophysin (mAB C7.2). Some coverslips, after single immunolabeling for synaptophysin, were incubated with fluorescein-conjugated wheat germ agglutinin (WGA). Cells were examined with a Zeiss Axiophot microscope and photographed using Tmax 100 film and developed with Tmax developer (Kodak, Rochester, NY).

Electron microscopy

Cells grown in 100 mm dishes were fixed in 3% formaldehyde, 0.05% glutaraldehyde, 0.15 M Na-phosphate pH 7.4 for 1 h on ice. For the horseradish peroxidase uptake experiments, cells were incubated for 1 h at 37°C in medium containing 10 mg/ml horseradish peroxidase (Type II, Sigma) prior to fixation. Cells were then washed and incubated for a further 15 min on ice in DMEM without peroxidase. After fixation, the peroxidase reaction product was developed as described (De Camilli *et al.*, 1986). The fixed peroxidase treated or untreated cells were rinsed with phosphate buffer, scraped with a rubber policeman and pelleted in an Eppendorf tube. The pellets were cut into small blocks which were then processed according to the method of Tokuyasu (1986; personal communication). Briefly, tissues were infiltrated for 1–2 h in 50% polyvinylpyrrolidone (PVP-10, Sigma, MO) containing 2.3 M sucrose in 0.1 M Na-phosphate buffer, pH 4, mounted on metal nails and frozen in liquid nitrogen. Ultrathin frozen sections were cut on glass knives with an Ultracut microtome equipped with an FC4 attachment (Reichert, Vienna). Sections were placed on glow-discharged, formvar- and carbon-coated nickel grids with a drop of 2.3 M sucrose in PBS. Imunogold labeling was performed essentially as described (Navone *et al.*, 1986) using gold conjugates of goat anti-rabbit IgGs or Protein A at the second labeling step. After the glutaraldehyde fixation, grids were processed through the following steps: brief rinses in water, 15 min in 2% OsO_4, brief rinse in water, 30 min in 2% uranyl acetate, 2 × 2.5 min in 2.3% polyvinylalcohol containing 15 μg/ml lead citrate. Finally, grids were air dried after removing excess fluid (Keller *et al.*, 1984; Tokuyasu, 1986, personal communication). For conventional plastic embedding and sectioning, cells were fixed in 2% glutaraldehyde, 0.12 M Na-phosphate pH 7.4, scraped, pelleted as described above and Epon embedded before sectioning.

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