

Synthesis of the Mammalian Synaptic Vesicle Protein Synaptophysin in Insect Cells: A Model for Vesicle Biogenesis

UWE LEIMER, WERNER W. FRANKE, AND RUDOLF E. LEUBE¹

Division of Cell Biology, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Federal Republic of Germany

The N-glycosylated integral membrane protein synaptophysin is one of the major polypeptide components of small presynaptic transmitter-containing vesicles in neurons and of similar vesicles in neuroendocrine cells of mammals. Functional properties, including a possible participation in channel formation, have been investigated by integration of purified synaptophysin into planar lipid bilayers. To overcome some of the inherent limitations of such an *in vitro* approach we have overexpressed the rat synaptophysin cDNA in nonneuronal, non-neuroendocrine insect cells with the help of recombinant baculovirus. The complete polypeptide was produced in infected ovarian Sf9 cells at levels exceeding those observed in rat brain. The partially N-glycosylated molecules could be extracted from membranes with non-ionic detergents, most effectively with *n*-octyl- β -D-glucopyranoside, and could be enriched on chromatofocusing columns. By immunoelectron microscopy synaptophysin was shown to be integrated in the correct orientation into the endoplasmic reticulum, various pleomorphic vesicles and the plasma membrane. Using cell fractionation, including density gradient centrifugation and immunoisolation, we characterized distinct synaptophysin-rich vesicles. These vesicles may help to understand molecular principles of vesicle biogenesis in general and the function of synaptophysin in particular.

© 1996 Academic Press, Inc.

INTRODUCTION

The biogenesis and repetitive recycling of presynaptic transmitter-containing vesicles must be controlled by extremely reliable and efficient mechanisms in order to maintain an extraordinary compositional specificity while allowing rapid exocytotic transmitter release and reformation of functional synaptic vesicles. We are trying to understand the role of synaptophysin in the life cycle of these vesicles as it has been identified as one of their major integral membrane components [1, 2].

¹ To whom correspondence and reprint requests should be addressed. Fax: ++49-6221-423404.

Synaptophysin belongs to a group of related polypeptides that includes, in addition to the other neuron-specific member synaptoporin (also referred to as synaptophysin II), the ubiquitously expressed pantophysin [3–7]. Common features of these polypeptides are four highly conserved hydrophobic, putative transmembrane domains, variable cytoplasmic termini, and intravesicular loops containing conserved cysteine residues and an N-glycosylation site [3–9]. It has been shown that synaptophysin and synaptoporin form homooligomers [6, 9, 10] and that synaptophysin is phosphorylated on serine and tyrosine residues [11–13].

Reagents specific for synaptophysin are reliable markers for neuronal and neuroendocrine (NE)² differentiation as it is an obligatory component of small electron-translucent (SET) secretory vesicles in these cell types [2, 14–17] suggesting a crucial contribution of this molecule to the biogenesis and function of such specialized membrane structures. This notion is further supported by the observations that synaptophysin induces the formation of a distinct SET vesicle type in cDNA-transfected epithelial cells [8, 18; for results in other cells see 16, 17, 19, 20], that purified synaptophysin forms voltage-sensitive pores in lipid bilayers [10], that synaptophysin antisense oligonucleotides or synaptophysin antibodies inhibit transmitter release [21, 22], and that a large proportion of synaptophysin is tightly associated with the v-SNARE synaptobrevin [23–25]. On the other hand, ablation of synaptophysin in mice [26] does not result in significant phenotypic alterations.

We describe now the expression of synaptophysin in ovarian-derived insect cells with the help of recombinant baculovirus and report the purification of a distinct synaptophysin-rich vesicle type from these cells. This cell system should be a valuable tool to examine the role of synaptophysin and synaptophysin mutants

² Abbreviations used: BRSY, recombinant baculovirus containing the rat synaptophysin cDNA; hpi, hours after infection; kb, kilobase pairs, i.e., 1000 bp; NE, neuroendocrine; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SET, small electron translucent; TCA, trichloroacetic acid.

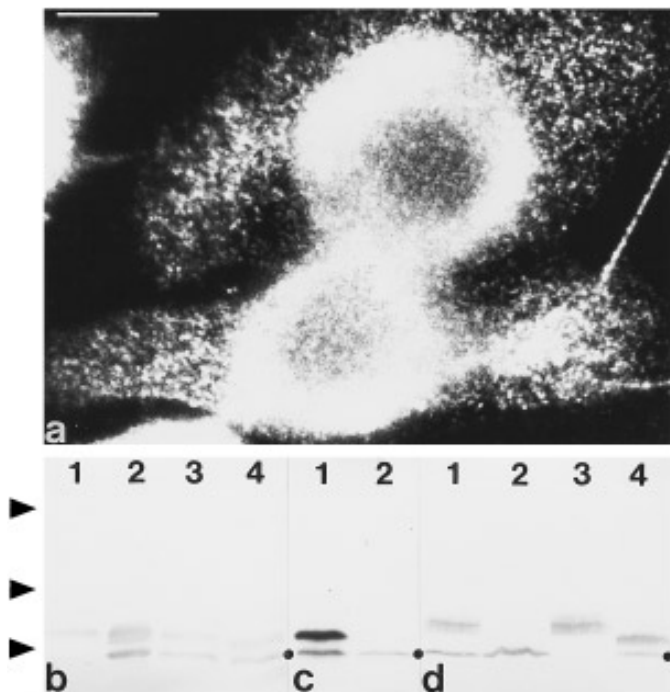


FIG. 1. Detection of synaptophysin in insect cells infected with the synaptophysin gene containing baculovirus BRSY. (a) Immunofluorescence microscopy using monoclonal antibody SY38 and Texas red-coupled secondary antibodies. Cells were grown for 36 hpi on poly-L-lysine-coated glass coverslips and fixed with methanol/acetone. Note the multipunctate cytoplasmic staining with pronounced perinuclear accumulation. Bar, 2 μ m. (b) Immunoblot detection of synaptophysin in postnuclear supernatant fractions obtained from rat brain (lane 1), from Sf9 cells either 72 hpi (lane 2) or 48 hpi (lane 3), and from High-5 cells 48 hpi (lane 4). Proteins (20 μ g in each lane) were separated by 12% SDS-PAGE and synaptophysin was detected by monoclonal antibody SY38 and the alkaline phosphatase system. (c, d) Detection of synaptophysin produced either *in vitro* by transcription-translation in the presence (c, lane 1) and absence of pancreatic microsomes (c, lane 2) or synthesized *in vivo* from BRSY-infected Sf9 cells (48 hpi; 20 μ g protein from S1 fractions in lanes 1, 2 of d) and from the endogenous synaptophysin gene in NE PC12 cells (20 μ g protein from S1 fractions in lanes 3, 4 of d). Cells were grown in either the absence (d, lanes 1 and 3) or presence (d, lanes 2, 4) of 1 μ g/ml tunicamycin 24 h prior to harvesting. Products were visualized by autoradiography (c) or by immunostaining of postnuclear supernatant fractions that were separated by 12% SDS-PAGE and reacted after electrotransfer onto nitrocellulose with monoclonal antibody SY38 and alkaline phosphatase-coupled secondary antibodies using the alkaline phosphatase detection system (d). The relative positions of coelectrophoresed size markers are indicated by arrowheads on the left margin of b (from top to bottom BSA, M_r 66,000; ovalbumin, M_r 45,000; glyceraldehyde-2-phosphate dehydrogenase, M_r 36,000). Note that the aglycon comigrates in all instances (dot).

for the biogenesis and functional properties of special cytoplasmic vesicles.

MATERIALS AND METHODS

Cell lines. NE rat pheochromocytoma cells of line PC12 (ATCC CRL 1721) were cultured as described [15]. Insect cells from either

Spodoptera frugiperda [lines Sf9 (kindly provided by Hanswalter Zentgraf, German Cancer Research Center, Heidelberg, FRG) and High 5 (Invitrogen, San Diego, CA) or *Trichoplusia ni* [line Sf21 (Invitrogen)] were grown at 28°C in TNM-FH medium (Sigma, St. Louis, MO) that was supplemented with 10% fetal calf serum (ICN Biomedicals GmbH, Meckenheim, FRG). Cells were passaged as monolayers in plastic vessels under standard conditions [27, 28]. In some instances tunicamycin (Sigma) was added to cultures at 1 μ g/ml for up to 2 days.

Production of recombinant baculovirus and infection of cultured insect cells. The rat synaptophysin cDNA was excised from plasmid pSR⁵ [8] by linearization first with *Hind*III and blunt end formation using T4 DNA polymerase followed by restriction with *Not*I. The 1-kb insert fragment was cloned next to the polyhedrin promoter of the transfer vector pVL 1393 (Invitrogen) that was linearized with *Bam*HI, treated with T4 DNA polymerase, cleaved with *Not*I, and dephosphorylated with calf intestinal phosphatase. Two micrograms of purified DNA of the resulting plasmid pBSR1 were cotransfected with 1 μ g wild-type baculovirus DNA (*Autographa californica* nuclear polyhedrosis virus; kindly provided by Hanswalter Zentgraf). The DNAs were suspended in 0.75 ml buffer (25 mM Hepes (pH 7.1), 140 mM NaCl, 25 mM CaCl₂) and added dropwise to 1×10^6 Sf9 cells during their logarithmic growth phase in a 12-cm² petri dish with 0.75 ml culture medium. After 4 h incubation at 28°C cells were washed two times with TNM-FH medium and grown for another 5–8 days until multiple nuclear polyhedra and extensive cell lysis were visible. The culture supernatant was saved and tested for the presence of virus that had taken up the synaptophysin gene by recombination with the polyhedrin gene. Therefore, virus-containing supernatants were diluted 10⁻² to 10⁻⁸ in culture medium and added to Sf9 cells growing in wells (2×10^4 cells per well) of a 96-well culture dish. Three to 4 days after infection, culture supernatants were transferred to a new multiwell culture dish and stored at 4°C. The remaining cells in each well were lysed in 200 μ l 0.5 M NaOH, neutralized with 20 μ l 10 M ammoniumacetate, and 100 μ l of this solution was blotted onto nitrocellulose membranes using a dot blot apparatus (Schleicher & Schüll, Dassel, FRG). The filter-bound samples were hybridized under stringent conditions to the *Hind*III/*Bam*HI synaptophysin insert fragment of clone pSR⁵ [8] that had been radioactively labeled by random oligonucleotide-directed priming [29]. The

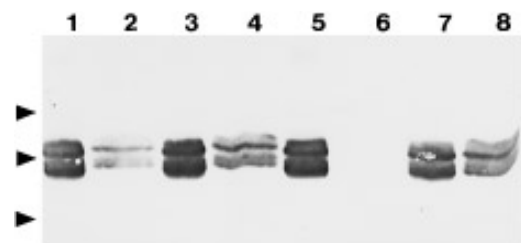


FIG. 2. Immunoblot detecting synaptophysin with monoclonal antibody SY38 and the alkaline phosphatase system in detergent-treated fractions from BRSY-infected Sf9 cells 72 hpi. Postnuclear supernatants were centrifuged at 100,000g for 1 h at 4°C and the pellet fractions were treated overnight with 1% Triton X-100 (lanes 1, 2), 1% NP-40 (lanes 3, 4), 1% *n*-octyl- β -D-glucopyranoside (lanes 5, 6), or 1% Chaps (lanes 7, 8). After extraction the solutions were recentrifuged at 100,000g for 1 h at 4°C and protein contained in equivalent amounts of either the resulting supernatant fractions (lanes 1, 3, 5, 7) or pellet fractions (lanes 2, 4, 6, 8) were separated by 12% SDS-PAGE prior to immunoblotting. Positions of coelectrophoresed size markers are shown on left by arrowheads (from top to bottom: ovalbumin, M_r 45,000; glyceraldehyde-2-phosphate dehydrogenase, M_r 36,000; carbonic anhydrase, M_r 29,000).

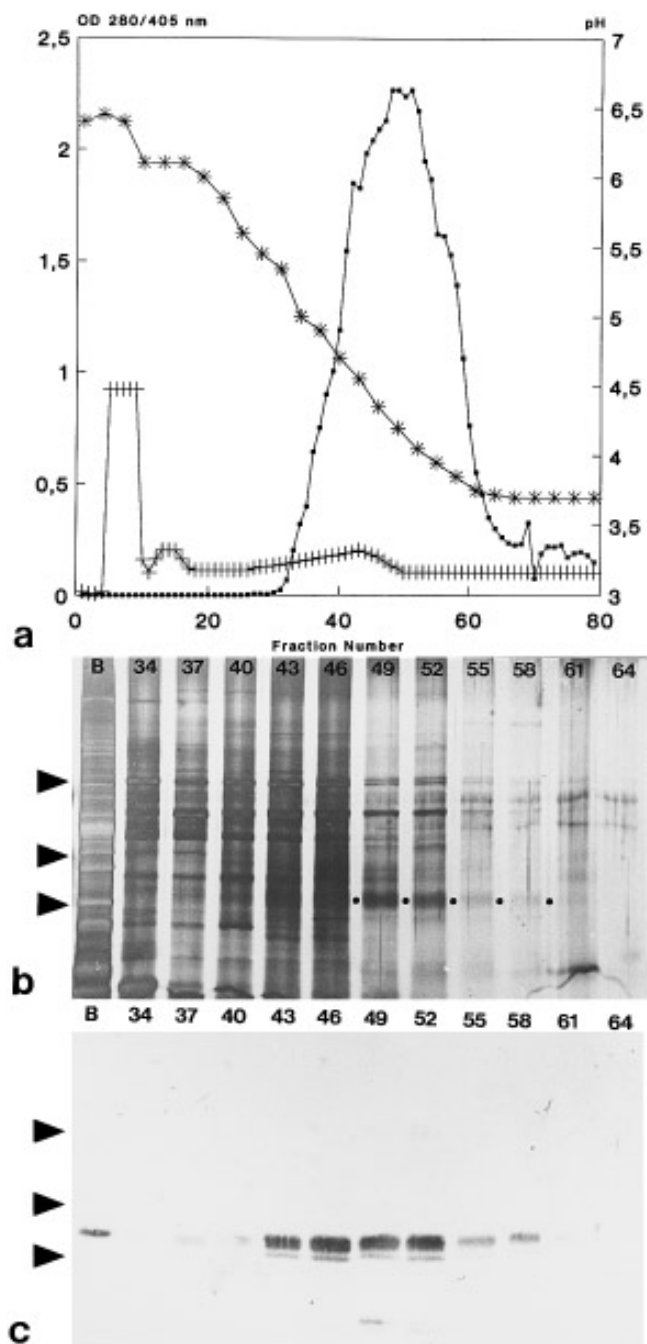


FIG. 3. Detection of synaptophysin in eluates from a chromatofocusing column. Proteins contained in $100,000g$ pellets of S1 fractions from BRSY-infected Sf9 cells (72 hpi) were solubilized in 1% Triton-X 100 and the resulting nonpelletable material was applied to a chromatofocusing column. (a) ELISA detecting synaptophysin immunoreactivity (absorption measured at 405 nm on left ordinate, squares) in eluting fractions. Absorption of the eluate was monitored at 280 nm with a flow through photometer (+, left ordinate with arbitrary scale) and the pH was determined in every fourth 2.5-ml fraction (stars; right ordinate). Note that the bulk of synaptophysin is detected in the pH range 4.8 to 3.8. (b) Silver staining of polypeptides contained in selected synaptophysin-containing peak fractions of a chromatofocusing column (see a) after separation by SDS-PAGE.

supernatants from cells showing a positive hybridization signal were subjected to further rounds of dilution and hybridization until no polyhedrin bodies were seen in infected cells. These supernatants containing recombinant virus BRSY were used for large-scale production of synaptophysin in infected insect cells.

Immunofluorescence and immunoelectron microscopy. Immunolocalization of synaptophysin by monoclonal antibody SY38 [2] or affinity-purified synaptophysin antibodies from rabbit [18] by immunofluorescence microscopy of methanol/acetone-treated cells and immunoelectron microscopy has been described [2, 18]. For electron microscopy detergent treatment was omitted in some instances of partially lysed cells.

Subcellular fractionation of synaptophysin-containing membranes. Cells or minced small tissue pieces were lysed in hypotonic buffer H (10 mM triethanolamine acetic acid (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.1 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride) and homogenized in a tight-fitting Dounce homogenizer by 20 to 30 up and down strokes. Centrifugation at $800g$ for 5 min yielded postnuclear supernatant S1. For further enrichment of synaptophysin-containing vesicles S1 supernatant [500 μ g protein as determined with the Bradford reagent (Bio-Rad, Hercules, CA)] was loaded onto an 11-ml linear sucrose gradient (15–60% in buffer H). After centrifugation at $270,000g$ at 4°C for 160 min in a SW 40 rotor (Beckman, Palo Alto, CA), 600- μ l fractions were collected from top to bottom. Synaptophysin content was determined by ELISA as described recently [18, 28].

For analysis of detergent solubility of synaptophysin, postnuclear S1 supernatants were centrifuged at $100,000g$ for 1 h at 4°C and the resulting pellets were treated with different detergents (Triton X-100 (Serva, Heidelberg, FRG), NP-40 (Fluka, Buchs, Switzerland), *n*-octyl- β -D-glucopyranoside (Sigma), Chaps (Sigma)) each at 1% (w/v) in buffer H supplemented with protease inhibitors (2 μ g/ml E64 (Sigma), 40 μ g/ml bestatin (Boehringer), 1 μ M pepstatin (Boehringer), and 0.2 mM phenylmethylsulfonyl fluoride) overnight at 4°C under constant agitation. The solution was again centrifuged at $100,000g$ for 1 h at 4°C , and supernatant and pellet fractions were analyzed by SDS-PAGE and immunoblotting [cf. 8, 18].

Synaptophysin-containing vesicles were immunoisolated from cells that were metabolically labeled [1 h, 200 μ Ci Tran ^{35}S -Label (ICN Biomedicals GmbH) with 6 ml methionine-deficient medium (Excell 401 from Sera-lab, Crawley Down, UK) per 10-cm-diameter petri dish]. Approximately 4×10^6 magnetic particles coated with sheep anti-mouse IgG1 (Dynabeads M-280; Dynal, Hamburg, FRG) were preadsorbed for 1 h in 5 ml isolation buffer IB (PBS containing 10 mM MEGTA, 0.1% BSA, and 10 mg/ml L- α -phosphatidylcholine type XVI-E from Sigma) supplemented with 1 ml postnuclear supernatant of noninfected, nonlabeled Sf9 cells, washed three times with IB, and coupled to monoclonal synaptophysin antibody SY38 [2] by incubation with 2 ml SY38 hybridoma together with 2 ml IB for 1–2 h. After several washes in IB, beads were resuspended in 5 ml IB, mixed with 2.5 ml of synaptophysin-containing sucrose gradient fractions (see above) that had been prepared from labeled cells, and incubated for 1 h. Immune complexes were washed several times with IB, including a short high-salt wash with 1 M KCl in IB, and the immunisolated material was analyzed by immunoblotting and autoradiography after SDS-PAGE.

Chromatofocusing. Postnuclear S1 supernatants were prepared (see above) and centrifuged at $100,000g$ for 1 h at 4°C . The resulting

(c) Immunoblot detection of synaptophysin in fractions corresponding to those shown in (b) by monoclonal antibody SY38 and the alkaline phosphatase system. Arrowheads in (b, c) show position of coelectrophoresed size markers (from top to bottom: M_r 67,000; 45,00; 36,000; cf. Fig. 1); B in (b, c) rat brain postnuclear supernatant fraction.

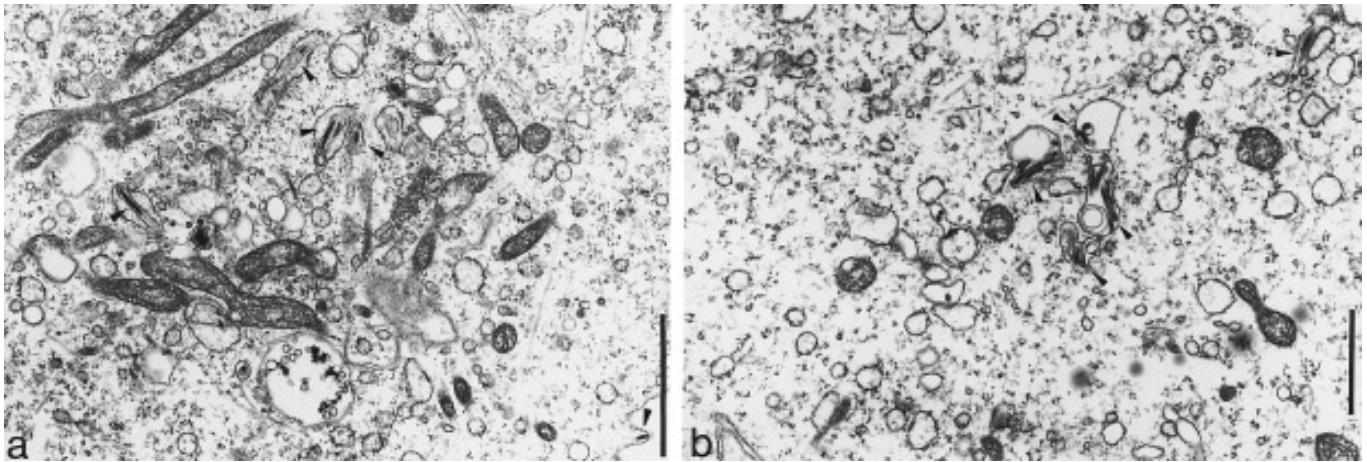


FIG. 4. Survey electron micrographs of representative cytoplasmic regions in Sf9 cells 36 h after infection with either recombinant, synaptophysin-expressing baculovirus BRSY (a) or with wild-type baculovirus (b). Note the presence of virus particles (arrowheads) and multiple vesicles of various shape and size in both micrographs. Bars, 1 μ m.

pellet was resuspended and proteins were solubilized by shaking overnight at 4°C in column loading buffer [25 mM sodiumsuccinate (pH 6.2), 2 mM EDTA, 2 mM EGTA, 2 mM DTT] supplemented with 1% Triton X-100. The solution was again centrifuged at 100,000g for 1 h at 4°C and 7 ml of the supernatant (3 mg protein/ml) were applied to a 0.7 \times 17 cm chromatofocusing column (Polybuffer Exchanger 94, Pharmacia, Uppsala, Sweden) that had been equilibrated with 200 ml column loading buffer containing 1% Triton X-100. Polypeptides were eluted with Polybuffer 74-HCL, pH 3.8 (Pharmacia), that had been diluted 1:8 in H₂O in the presence of 0.1% (w/v) Triton X-100 and protease inhibitors (see above). Fractions (2.5 ml) were collected and tested for their relative content of synaptophysin by ELISA. Polypeptides were concentrated by TCA precipitation [cf. 18], separated by SDS-PAGE, and detected by silver staining [30].

In vitro transcription and translation. *In vitro* transcription and translation was performed as described using T3 RNA polymerase and *Hind*III-linearized plasmid pSR⁵ [8]. In some experiments pancreatic microsomes (Promega) were added to the *in vitro* translation reaction following the procedures suggested by the manufacturer. The products were separated by SDS-PAGE and labeled proteins were visualized by autoradiography of dried gels.

RESULTS AND DISCUSSION

Overexpression of rat synaptophysin in insect cells. To examine the distribution of synaptophysin in nonepithelial, non-NE ovarian insect cells we constructed and produced the synaptophysin gene-containing baculovirus BRSY that was enriched by several rounds of dilution and nucleic acid screening of infected Sf9 cells. In immunofluorescence microscopy a strong perinuclear staining was noticed that could be resolved as multiple cytoplasmic dots in the cell periphery of BRSY-infected cells (Fig. 1a). For optimization of expression, several insect cell lines were tested for synaptophysin content after infection with BRSY at different time points. The immunoblot in Fig. 1b shows that the highest synaptophysin level was detectable two days after infection in Sf9 cells (Fig. 1b, lane 2) which was severalfold higher

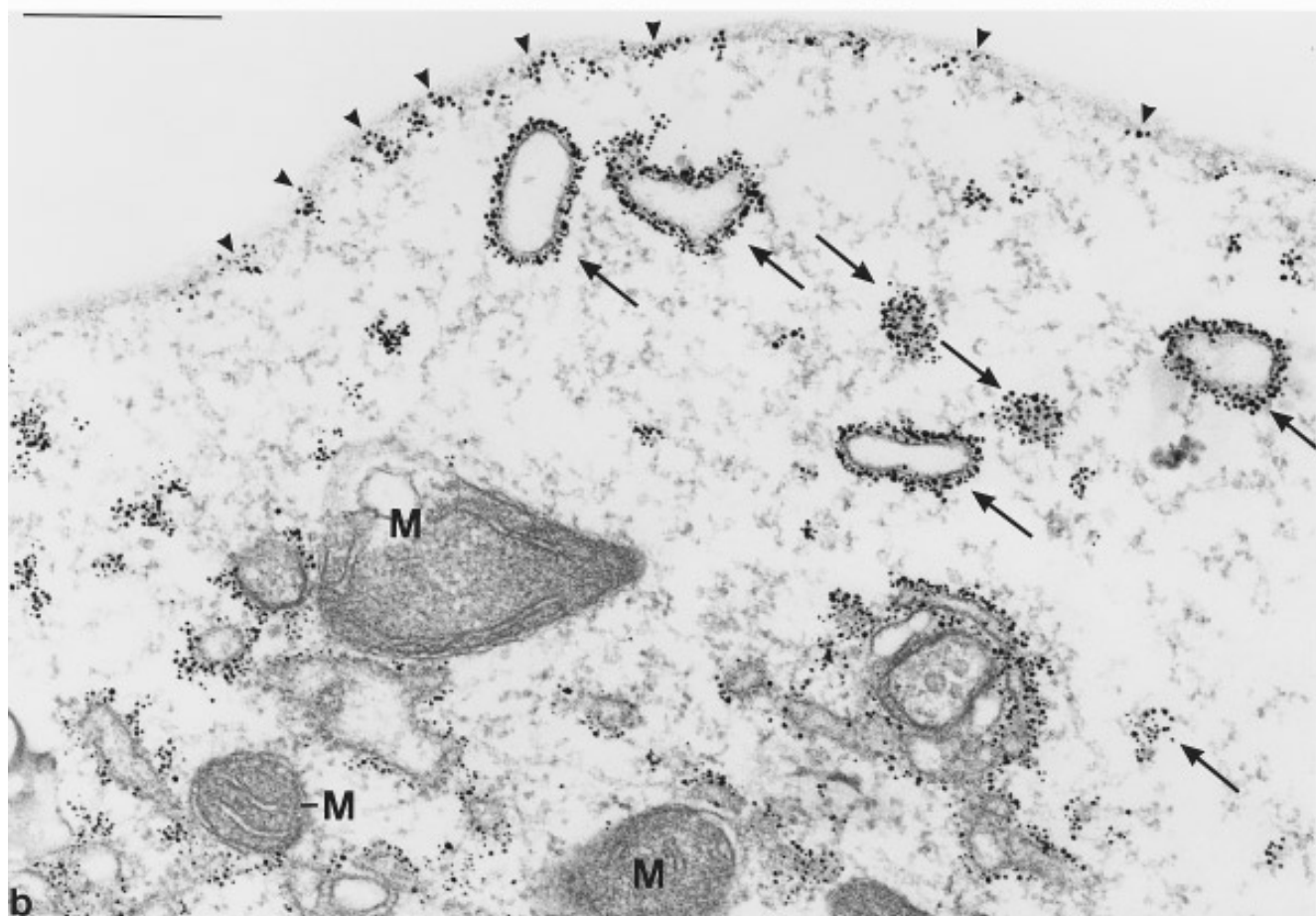
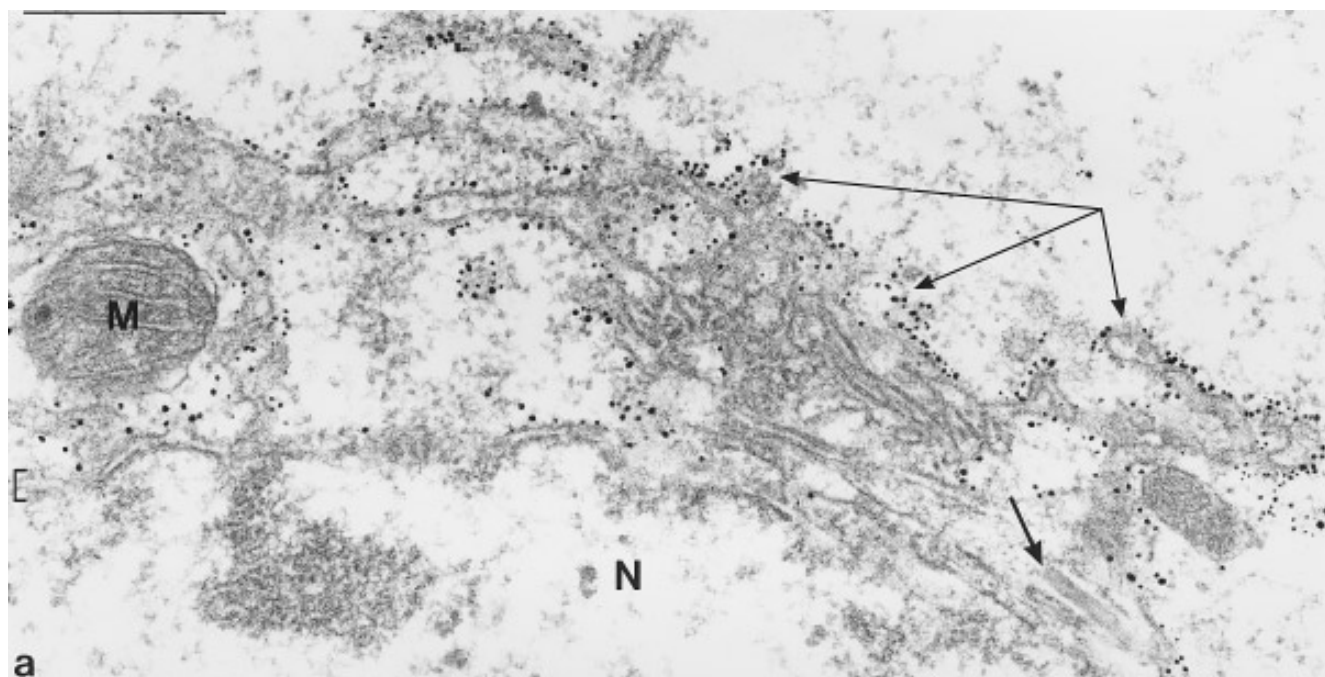
than that seen in rat brain homogenates (compare Fig. 1b, lanes 1 and 2).

Biochemical characterization and enrichment of synaptophysin produced in Sf9 cells. In insect cells infected with recombinant baculovirus BRSY several immunoreactive polypeptide bands were detected in immunoblot analyses (Fig. 1b) which could be attributable to posttranslational modification. Therefore, infected cells and NE PC 12 cells were treated in parallel with tunicamycin resulting in the disappearance of the higher-molecular-weight immunoreactive bands and the concomitant increase of a lower-molecular-weight species comigrating with synaptophysin generated *in vitro* from the cloned cDNA (compare Figs. 1c and 1d). This demonstrates that a significant proportion of synaptophysin is N-glycosylated in infected insect cells.

To further examine the biochemical properties of synaptophysin produced in insect cells, membrane pellets were prepared from postnuclear supernatants from BRSY-infected cells and treated with various non-ionic detergents. The comparison of several detergents showed that synaptophysin could be extracted from membranes most efficiently with *n*-octyl- β -D-glucopyranoside (Fig. 2).

By isoelectric focusing it has been estimated previously that synaptophysin has a very low pI of about 4.8 with several isoelectric variants [2]. We therefore fractionated solubilized synaptophysin from infected insect cells on a chromatofocusing column and could show that it elutes in the expected pH range between 3.8 and 4.8 (Fig. 3a). This procedure could be used to enrich synaptophysin sufficiently to detect it by silver staining (Fig. 3b and corresponding immunoblot in Fig. 3c).

Microscopical localization of synaptophysin in infected insect cells. Sf9 cells infected either with BRSY



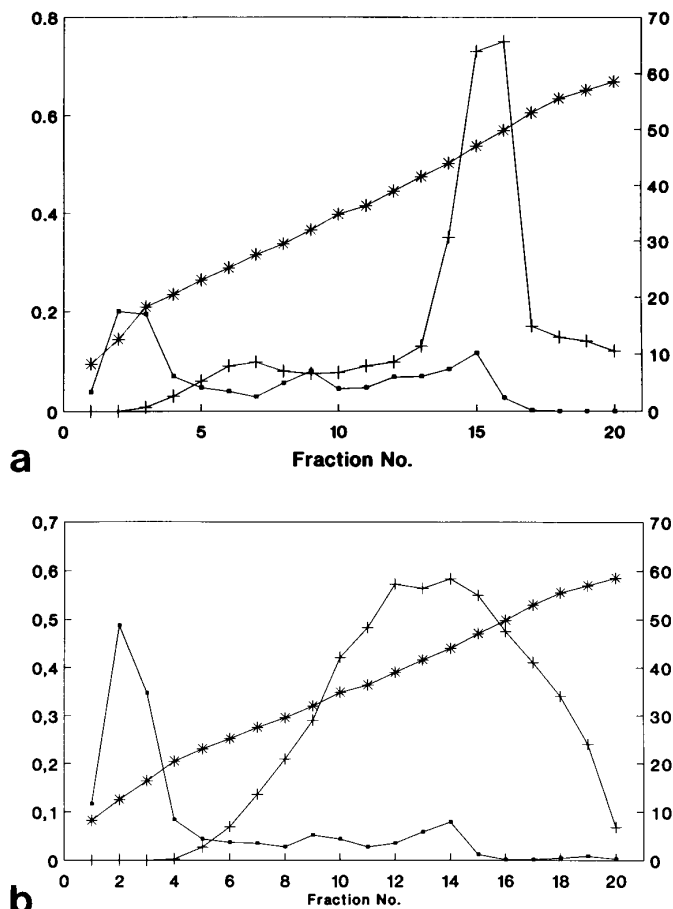


FIG. 6. Comparison of synaptophysin distribution in sucrose gradient fractions of BRSY-infected Sf9 cells 72 hpi (a) and NE PC12 cells (b) by ELISA. Postnuclear (800g) supernatants were layered on top of 15–60% linear sucrose gradients. After centrifugation at 270,000g for 160 min in a SW 40 Ti rotor (Beckman) 600- μ l fractions were collected from top (fractions numbered 1) to bottom (fractions numbered 20) and absorbance at 280 nm was recorded (squares; an arbitrary scale is given on left ordinates). The sucrose concentration in each fraction was determined with an Abbé refractometer (stars; given here as w/v % on the right ordinates). The relative synaptophysin concentration was determined with an ELISA measuring the absorbance at 405 nm (+; scales on left ordinates denote observed ODs at 405 nm).

or with wild-type baculovirus were compared by electron microscopy (Fig. 4). In both instances many vesicular profiles of different diameter were seen in the cyto-

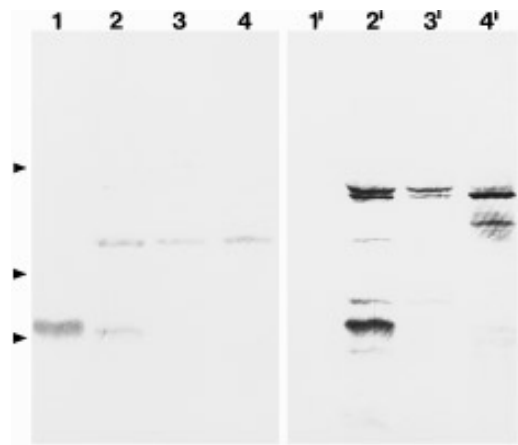


FIG. 7. Detection of synaptophysin in immunisolated vesicles. Subconfluent Sf9 cells (three 10-cm culture dishes in each experiment) were infected with either recombinant virus BRSY (lanes 2 and 2') or wild-type Baculovirus (lanes 3 and 3') or not infected at all (lanes 4 and 4'). Cells were metabolically labeled with 300 μ Ci Tran³⁵S-Label per culture dish for 1 h in methionine-deficient medium and synaptophysin-containing vesicles were isolated from sucrose gradient fractions 14 and 15 (see Fig. 6a) with SY38 antibody bound to magnetic beads. The adsorbed polypeptides were separated by 12% SDS-PAGE, blotted onto nitrocellulose, and again reacted with monoclonal antibody SY38. Lane 1 contains postnuclear supernatant from rat brain as a positive control. Note the detection of synaptophysin only in cells infected with recombinant BRSY (lane 2). The upper bands show the immunoglobulin heavy chains detected by the secondary antibody. The position of the molecular weight markers of M_r 66,000, 45,000, and 36,000 (for details see Fig. 1b) are indicated on the left. The immunoblot was then subjected to autoradiography (lanes 1'–4'). Note abundance of synaptophysin in lane 2' which corresponds exactly to the synaptophysin immunosignal in lane 2. Most of the other labeled molecules were adsorbed nonspecifically to the particles as they are also seen in the negative controls (lanes 3' and 4').

plasm with no apparent enrichment of a particular vesicle type. To identify the synaptophysin-containing vesicles, immunoelectron microscopy was performed on BRSY-infected insect cells and antibody gold label was found in certain locations (Figs. 5a and 5b): The ribosome-studded membranes of the rER cisternae were decorated by gold particles (Fig. 5a), and abundant synaptophysin immunoreactivity was consistently detected on the cytoplasmic side of smoothly surfaced vesicles of various sizes (arrows in Fig. 5b) and in certain patterns on the inner aspect of the plasma membrane (arrowheads in Fig. 5b).

FIG. 5. Immunoelectron microscopy of Sf9 cells 36 hpi with recombinant baculovirus BRSY, detecting synaptophysin with affinity-purified synaptophysin antibodies from rabbit and 5-nm gold-coupled secondary antibodies, together with the silver amplification method. Cells were fixed with 2.5% formaldehyde and either treated with 0.1% saponin (a) or not (b).

Note the abundant presence of synaptophysin in different membranes. The localization in cisternae of the rough endoplasmic reticulum is denoted by the triple arrow in (a). The bracket indicates the position of the nuclear envelope which also contains some immunolabel. No signal is seen inside the nucleus (N) or in mitochondria (M). Arrow in (a) denotes empty nucleocapsid in proximity to the nucleus. Larger vesicles and/or membrane invaginations are shown in (b) by arrows. Immunoreactivity in structures close to the plasma membrane is indicated by arrowheads. Note also the presence of silver grains in smaller cytoplasmic vesicles. Bars: (a) 250 nm, (b) 500 nm.

Isolation of synaptophysin-rich vesicles from BRSY-infected insect cells. Sucrose gradient centrifugation of postnuclear supernatant fractions was performed to enrich synaptophysin-containing cytoplasmic vesicles. ELISA and immunoblot analyses showed that synaptophysin immunoreactivity was markedly enriched in fractions with a density between 42 and 52% sucrose, respectively (Fig. 6a). A similar, albeit somewhat less restricted, distribution was noted for synaptophysin vesicles extracted from NE PC12 cells (Fig. 6b). The fractions containing most synaptophysin immunoreactivity were pooled and used in immunoisolation experiments with antibody-coated magnetic beads. In these experiments cells were metabolically labeled with [³⁵S]-methionine to determine the polypeptide composition of the immunoadsorbed vesicles. The major polypeptide component detected by autoradiography of the electrophoretically separated polypeptides was synaptophysin (Fig. 7).

Conclusions. We have constructed recombinant baculovirus for the high-level expression of synaptophysin in insect cells. In contrast to bacterial systems posttranslational modification took place and overexpression was possible. Although we were able to obtain relative synaptophysin levels that were severalfold higher than those in rat brain the ectopically produced polypeptides were only minor components of the total cellular proteins which were, however, comparable to the reported amounts of other polytopic membrane proteins produced in insect cells [e.g., 31–34]. The usefulness of BRSY-driven expression of synaptophysin in insect cells is underlined by the observations that synaptophysin was incorporated into membranes in the proper orientation [compare with 3, 8, 9, 16–18], that a significant proportion of synaptophysin was modified by attachment of carbohydrate moieties to asparagine residues [for different degrees of glycosylation see also 2, 8, 14, 16, 35], and that the *pI* of synaptophysin was in good agreement with reported values for rat brain synaptophysin [2]. Most importantly, the multipunctate immunofluorescent distribution [compare with the similar patterns in other cells in 8, 14–20, 35, 36] was in part due to the formation of a distinct synaptophysin-rich vesicle type. These vesicles produced in a living cell should facilitate further functional analyses, thereby extending *in vitro* reconstitution experiments [10] with their limitations concerning membrane integration and formation of functional membrane domains. The detailed analysis of synaptophysin and selected synaptophysin mutants (see, e.g., [36]) produced in these cells will be a useful model for the investigation of the importance of a single molecule for the formation of a distinct vesicular membrane domain with specific functional properties.

We are grateful for the skillful work of Anke Marschall, Judith

Rudisile, and Christine Grund and also thank Dr. Hanswalter Zentgraf for many useful tips and help in the establishment of the baculovirus expression system. The work was supported by the German Research Council (SFB 317).

REFERENCES

- Jahn, R., Schiebler, W., Ouimet, C., and Greengard, P. (1985) *Proc. Natl. Acad. Sci. USA* 82, 4137–4141.
- Wiedenmann, B., and Franke, W. W. (1985) *Cell* 41, 1017–1028.
- Leube, R. E., Kaiser, P., Seiter, A., Zimbelmann, R., Franke, W. W., Rehm, H., Knaus, P., Prior, P., Betz, H., Reinke, H., Beyreuther, K., and Wiedenmann, B. (1987) *EMBO J.* 6, 3261–3268.
- Südhof, T. C., Lottspeich, F., Greengard, P., Mehl, E., and Jahn, R. (1987) *Science* 238, 1142–1144.
- Knaus, P., Marquèze-Pouey, B., Scherer, H., and Betz, H. (1990) *Neuron* 5, 453–462.
- Fykse, E. M., Takei, K., Walch-Solimena, C., Geppert, M., Jahn, R., De Camilli, P., and Südhof, T. C. (1993) *J. Neurosci.* 13, 4997–5007.
- Leube, R. E. (1994) *Differentiation* 56, 163–171.
- Leube, R. E., Wiedenmann, B., and Franke, W. W. (1989) *Cell* 59, 433–446.
- Johnston, P. A., and Südhof, T. C. (1990) *J. Biol. Chem.* 265, 8869–8873.
- Thomas, L., Hartung, K., Langosch, D., Rehm, H., Bamberg, E., Franke, W. W., and Betz, H. (1988) *Science* 242, 1050–1052.
- Pang, D. T., Wang, J. K. T., Valtorta, F., Benfenati, F., and Greengard, P. (1988) *Proc. Natl. Acad. Sci. USA* 85, 762–766.
- Barnekow, A., Jahn, R., and Schartl, M. (1990) *Oncogene* 5, 1019–1024.
- Rubenstein, J. L., Greengard, P., and Czernik, A. J. (1993) *Synapse* 13, 161–172.
- Navone, F., Jahn, R., Di Gioia, G., Stukenbrok, H., Greengard, P., and De Camilli, P. (1986) *J. Cell Biol.* 103, 2511–2527.
- Franke, W. W., Grund, C., and Achstätter, T. (1986) *J. Cell Biol.* 103, 1933–1943.
- Johnston, P. A., Cameron, P. L., Stukenbrok, H., Jahn, R., De Camilli, P., and Südhof, T. C. (1989) *EMBO J.* 8, 2863–2872.
- Cameron, P. L., Südhof, T. C., Jahn, R., and De Camilli, P. (1991) *J. Cell Biol.* 115, 151–164.
- Leube, R. E., Leimer, U., Grund, C., Franke, W. W., Harth, N., and Wiedenmann, B. (1994) *J. Cell Biol.* 127, 1589–1601.
- Linstedt, A. D., and Kelly, R. B. (1991) *Neuron* 7, 309–317.
- Feany, M. B., Yee, A. G., Delvy, M. L., and Buckley, K. M. (1993) *J. Cell Biol.* 123, 575–584.
- Alder, J., Lu, B., Valtorta, F., Greengard, P., and Poo, M.-m. (1992) *Science* 257, 657–661.
- Alder, J., Xie, Z.-P., Valtorta, F., Greengard, P., and Poo, M.-m. (1992) *Neuron* 9, 759–768.
- Calakos, N., and Scheller, R. H. (1994) *J. Biol. Chem.* 269, 24534–24537.
- Edelmann, L., Hanson, P. I., Chapman, E. R., and Jahn, R. (1995) *EMBO J.* 14, 224–231.
- Washbourne, P., Schiavo, B., and Montecucco, C. (1995) *Biochem. J.* 305, 721–724.
- Eshkind, L. G., and Leube, R. E. (1995) *Cell Tiss. Res.*, in press.
- Summers, M. D., and Smith, G. E. (1987) *in* Bulletin No. 1555, Texas Agriculture Experiment Station, pp. 10–39, Texas A & M Univ., College Station, TX.

28. Leimer, U. (1993) Diploma thesis, Faculty of Biology, Univ. of Heidelberg, pp. 1–102.
29. Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* 132, 6–13.
30. Blum, H., Beier, H., and Gross, H. J. (1987) *Electrophoresis* 8, 93–99.
31. Baer, C. E., Canhui, L., Kartner, N., Bridges, R. J., Jensen, T. J., Ramjeesingh, M., and Riordan, J. R. (1992) *Cell* 68, 809–818.
32. Peng, S., Sommerfelt, M., Logan, J., Huang, Z., Julling, T., Kirk, K., Hunter, E., and Sorscher, E. (1993) *Protein Expression Purif.* 4, 95–100.
33. Keinänen, K., Köhr, G., Seeburg, P. H., Laukkanen, M.-L., and Oker-Blom, C. (1994) *Bio/Technology* 12, 802–806.
34. Li, M., Unwin, N., Stauffer, K. A., Jan, Y.-N., and Jan, L. Y. (1994) *Curr. Biol.* 4, 110–115.
35. Wiedenmann, B., Franke, W. W., Kuhn, C., Moll, R., and Gould, V. E. (1986) *Proc. Natl. Acad. Sci. USA* 83, 3500–3504.
36. Leube, R. E. (1995) *J. Cell Sci.* 108, 883–894.

Received October 19, 1995

Revised version received December 4, 1995