Sorting of Synaptophysin into Special Vesicles in Nonneuroendocrine Epithelial Cells

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Abstract. Synaptophysin is a major transmembrane glycoprotein of a type of small vesicle with an electron-translucent content (SET vesicles), including the ~50-nm presynaptic vesicles in neuronal cells, and of similar, somewhat larger (≤~90 nm) vesicles (SLMV) in neuroendocrine (NE) cells. When certain epithelial non-NE cells, such as human hepatocellular carcinoma PLC cells, were cDNA transfected to synthesize synaptophysin, the new molecules appeared in specific SET vesicles. As this was in contrast to other reports that only NE cells were able to sort synaptophysin away from other plasma membrane proteins into presynaptic- or SLMV-type vesicles, we have further characterized the vesicles containing synaptophysin in transfected PLC cells. Using fractionation and immunoisolation techniques, we have separated different kinds of vesicles, and we have identified a distinct type of synaptophysin-rich, small (30–90-nm) vesicle that contains little, if any, protein of the constitutive secretory pathway marker hepatitis B surface antigen, of the fluid phase endocytosis marker HRP, and of the plasma membrane recycling endosomal marker transferrin receptor. In addition, we have found variously sized vesicles that contained both synaptophysin and transferrin receptor. A corresponding result was also obtained by direct visualization, using double-label immunofluorescence microscopy for the endocytotic markers and synaptophysin in confocal laser scan microscopy and in double-immunogold label electron microscopy. We conclude that diverse non-NE cells of epithelial nature are able to enrich the "foreign" molecule synaptophysin in a category of SET vesicles that are morphologically indistinguishable from SLMV of NE cells, including one type of vesicle in which synaptophysin is sorted away from endosomal marker proteins. Possible mechanisms of this sorting are discussed.

INTERCELLULAR fast signal transduction is facilitated by exocytosis of neurotransmitter-containing, small (diameter = 30–90 nm) electron translucent (SET)[1] vesicles, of which the ~50 nm presynaptic vesicles (PSV) of neurons are the prototype. Despite the identification of an increasing number of molecules constituting the membrane of such vesicles (for review see reference 43), and despite numerous observations of vesicle exocytosis and recycling by endocytosis (e.g., 14, 21–23, 31, 32, 46, 48), the mechanisms of the formation of these organelles in the living cell are still unknown. Therefore, recent observations of compositionally similar, only somewhat larger (≤90 nm) and more pleiomorphic SET vesicles in neuroendocrine (NE) cells, which are now collectively referred to as "synaptic-like microvesicles" (SLMV), have initiated a series of studies of SET vesicle formation and turnover (e.g., 2–5, 28, 33, 37, 38, 45).

Particular attention has been paid to the synthesis and sorting of synaptophysin, the major transmembrane glycoprotein of PSV and of SLMV in different kinds of NE cells (e.g., 9, 26, 33, 39, 52, 54; for a recent review see 45). The highly conserved synaptophysin gene (25, 42) encodes a polypeptide of mol wt 33,312 (in rat) with four transmembrane domains that is N-glycosylated in the first intravesicular loop domain (26), can be phosphorylated in specific ways (35; for review see also reference 43), is sulfated on carbohydrate moieties (38), and is stabilized by intramolecular disulfide bonds (20). It forms homooligomers, which confer voltage-gated channel conductance after integration into lipid bilayers (20, 44). The specific forms of N-glycosylation and sulfation are commonly taken as an indication of its passage through the Golgi apparatus and the *trans*-Golgi network (38, 52), and the glycoprotein has indeed been detected by

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1. *Abbreviations used in this paper*: HBs, hepatitis surface antigen; NE, neuroendocrine; PSV, presynaptic vesicle(s); SET, small electron translucent; SLMV, synaptic-like microvesicles; TF, transferrin; TFR, transferrin receptor; TGN, *trans*-Golgi network.

immunoelectron microscopy in Golgi cisternae of neuronal and NE cells (37, 46; for support by other methods see 11). It has also been proposed that upon vesicle exocytosis, synaptophysin is transiently integrated into the plasma membrane, whereupon it is quickly recycled in the endocytotic compartment, defined by its ability to take up fluid-phase markers such as HRP and to coaccumulate with various recycling receptors such as transferrin receptor (TFR) and LDL receptor (e.g., 2, 19, 26, 28, 38, 45, 47; for a contrasting statement see reference 3). Unknown NE cell type-specific sorting mechanisms have then been postulated by several authors to effect the subsequent separation of synaptophysin from these receptors and other proteins, resulting in its concentrating in SET vesicles throughout the cytoplasm and their possible exocytotic rerelease to the cell surface (2, 21–23, 28, 38, 43).

By forced overexpression of synaptophysin in cultured non-NE cells transfected with synaptophysin gene constructs, it was shown that the "foreign" glycoprotein accumulated in certain small vesicles (cf. 19, 26, 28). However, the mode of formation and the nature of these vesicles has remained a matter of controversy. We have reported that in such transfected cells, a considerable proportion of synaptophysin is sorted away from other membrane proteins, and it is enriched in SLMV, either alone or in combination with other minor components (26). Other authors have concluded that specific sorting into PSV or SLMV is exclusive to NE cells, in which synaptophysin is sorted away from other endosomal membrane proteins (2, 3, 6, 21–23, 28). Since the latter concept predicts for all non-NE cells a complete codistribution of synaptophysin with endocytosed surface receptors, as well as the inability of sorting this glycoprotein into SLMV, we examined the intracellular distribution of these molecules in several transfected non-NE cell lines, using biochemical and immunolocalization methods.

Materials and Methods

Cell Culture

The following human cell lines were used: hepatocellular carcinoma-derived PLC cell sublines PLC (ATCC CRL 8024) and PLC-5S4 (26), as well as SK-Hep-1 cells (ATCC HTB 52), and vulvar carcinoma-derived A-431 cells (clone E_3; ATCC CRL 1555). For metabolic labeling, cells were grown in a culture dish (10 cm in diameter) to confluency, and they were then incubated either overnight (16 h) in growth medium with a reduced methionine content to which 150 μCi [^{35}S]methionine (Amersham International, Amersham, Bucks, U.K.) had been added, or for 1 h in Ex-Cell 40 medium (Sera-Lab Ltd., Sussex, U.K.). In some cases, cells were treated with 5 μg/ml brefeldin A (Sigma Immunochemicals, St. Louis, MO) for $\leq$3 h and/or 20 μg/ml cycloheximide (Sigma) for $\leq$8 h.

For comparison, NE cells of line PC12 were grown as described (9).

DNA Transfection

DNA transfection was done either by the calcium phosphate precipitation method (cf. 26) or by lipofection (7). In brief, plasmid DNA was purified by two cycles of cesium chloride centrifugation, and 25 μg DNA were dissolved in 450 μl H_2O. 50 μl of a 2.5-M $CaCl_2$ solution was added, and the whole mixture was dropwise pipeted into 500 μl 2× BES buffer (1× BES buffer is 140 mM NaCl, 0.75 M Na_2HPO_4, 50 mM *N,N-bis*-[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) adjusted to pH 7.05 with 1 N KOH) while vortexing. After incubation at room temperature for 20 min, the solution was added to a culture at low density (20–40% confluent) in a 10-cm culture dish. The cells were incubated with the precipitate overnight (16 h) under reduced CO_2 concentration (3%). After three washes with DME, cells were treated with 15% glycerol in 1× BES for 3 min and three washes with DME, followed by the addition of regular growth medium supplemented with penicillin-streptomycin (100 U/ml; Gibco, Karlsruhe, FRG). For selection of stably transfected cell clones, puromycin (Sigma) was added to a final concentration of 4 μg/ml, and single colonies were picked with a pipet (Gilson Medical Electronic Instruments, Middleton, WI) ~2 wk later. Expression was determined by immunofluorescence microscopy and Northern blotting. For increased expression of transgenes, transiently and stably transfected cells were sometimes treated with 5 mM sodium butyrate (Sigma) for 24 h (cf. 26).

For lipofection, 20 μg CsCl-purified plasmid DNA was dissolved in 100 μl H_2O, and 80 μg lipofectin reagent (BRL, Gaithersburg, MD) was diluted in a separate tube to 100 μl with H_2O. Both solutions were mixed gently in a polystyrene tube and incubated at room temperature for 15 min. Cells grown in 10-cm culture dishes to ~50% confluency were washed three times with serum-free medium. 10 ml serum-free medium was added, and the lipofectin reagent–DNA complexes were dropwise added while swirling the plates. After 16 h incubation, regular growth medium was added, and the cells were propagated and selected as described above.

To optimize the level of synaptophysin expression, several promoters in various plasmid constructs were tested. Best results were obtained with plasmid clone pSR[10], which contains the ~1 kbp KpnI/BamHI synaptophysin encoding insert of clone pSR[5] (26) in the eukaryotic expression vector pBEHpac18. This vector contains two separate SV-40 promoter/enhancer regions that drive the expression of any inserted foreign gene, as well as of the puromycin resistance gene (17).

Endocytosis of TFR, TF, and HRP

For TFR endocytosis, cells were washed three times with serum-free DME and were then incubated in serum-free DME for 1 h at 37°C. After several washes with ice-cold DME, cells were incubated at 4°C in serum-free DME containing TFR antibody B3/25 (Boehringer Mannheim GmbH, Mannheim, FRG) at a concentration of 1–10 μg/ml for 30 min. Addition of diferric TF (from Collaborative Research, Inc., Bedford, MA, or Bayer Diagnostic GmbH, München, FRG) did not significantly alter the results (cf. 1, 50), and it was therefore omitted in the later experiments. In some experiments, 15-nm gold particles coupled to sheep anti-mouse IgG or ^{35}S-labeled sheep anti-mouse IgG (Amersham International) were added in substoichiometric amounts at the same time. The resulting differences in half-life and cell surface expression of the TFR are not of relevance for this study since receptor recycling is only insignificantly altered (see reference 51). Accordingly, light microscopic controls did not show any differences in TFR distribution. After the adsorption at 4°C, FCS was added (5%), and cells were incubated for various time intervals of $\leq$2 h (in most cases, a 60-min incubation period was used, which is considerably longer than the reported time for saturation of intracellular TFR-containing organelles in other cell systems (e.g., 13, 18).

For transferrin (TF) endocytosis, cells were washed and preincubated as described above. Thereafter, cells were incubated with diferric TF at a concentration below receptor saturation (15 μg/ml) for 30 min at 37°C. Cells were washed extensively with PBS before further processing.

For endocytosis of HRP (type VI; Sigma), cells were incubated with HRP at a concentration of 10 mg/ml for various time intervals ranging from 5 min to 2 h (cf. 26). In biochemical analyses, peroxidase activity was determined by incubation of 0.2 ml of cellular fractions (usually obtained by sucrose density gradient centrifugation) with 0.8 ml of a freshly prepared assay solution containing 0.1 M sodiumcitrate (pH 5.5), 0.125% Triton X-100, 0.804 mM *o*-dianisidine, and 0.103 mM H_2O_2. Adsorption was measured at 450 nm exactly 30 min after mixing, and was compared to activities of known concentrations of HRP (0.05 ng, 0.1 ng, 0.2 ng).

Antibodies

Synaptophysin mAb SY38 (52) was used after purification by HPLC. Murine mAbs B3/25 (Boehringer Mannheim) and OKT9 (ATCC CRL8021; purified antibody was kindly provided by Dr. Rudolf Tauber (Free University of Berlin, FRG; cf. reference 34) against the human TFR were used at a dilution of 1:50 or 1:200, respectively, for immunofluorescence microscopy. Antibodies recognizing specifically human TF were from rabbits (Janssen Biochimica, Beerse, Belgium) or goats (Bio-Science Products AG, Emmenbrücke, Switzerland), and sheep antibodies against the hepatitis B virus surface antigen were obtained from Dako Diagnostika (Hamburg, FRG).

Polyclonal synaptophysin antibodies from rabbits (cf 9, 26) and guinea pigs were affinity purified on fusion proteins that had been produced in

Escherichia coli as follows. An ~250-bp long ThaI fragment encoding the carboxyterminus of synaptophysin was excised from clone pSR[5] (26) and inserted into the NruI site of the *E. coli* expression vector pAX5[+] (Medac, Hamburg, FRG). The plasmid was grown in *E. coli* (TG1) cells of a dense overnight culture that was diluted 1:10 (0.5 liter), and was further propagated at 25°C to an OD_{600} of 0.2. Fusion protein synthesis was induced by addition of isopropyl-thiogalactoside (final concentration = 1 μM) and a temperature shift to 37°C. After 2 h, cells were pelleted (5,000 *g*; 10 min at 4°C) and then resuspended in 20 ml ice-cold solution (0.5 M NaCl, 20 mM Tris-HCl, pH 7.5), 2 mM EDTA, 10 mM β-mercaptoethanol, 1 mM PMSF) containing 25 μl aprotinin (Sigma). Cells were lysed by repeated sonication, and the lysate was centrifuged for 1 h at 100,000 *g* and 4°C. The NaCl concentration of the resulting supernatant was adjusted to 1.6 M, and the solution was loaded onto a *p*-aminophenyl-β-D-thiogalactoside column (MoBiTec GmbH, Göttingen, FRG), preequilibrated with a buffer consisting of 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1.6 M NaCl, and 10 mM β-mercaptoethanol. The column was then extensively washed with this equilibration buffer, and bound polypeptides were eluted with 0.1 M di-sodium-tetraborate (pH 10.3) containing 10 mM β-mercaptoethanol. The pH of the eluate was neutralized by addition of HCl.

For affinity purification of antibodies, the purified fusion proteins were bound to nitrocellulose strips (1 mg/cm²). Filters were then blocked with 1% goat serum in PBS at 4°C overnight or at room temperature for 1 h. All tubes used for subsequent handling were pre-adsorbed in the same manner for ≤30 min at room temperature. Blocked nitrocellulose strips were incubated with antiserum (diluted in PBS) for at least 1 h. The supernatant was saved and could be used for another round of adsorption if it still showed reactivity. Filters were washed three times with PBS, and bound antibody was released by incubation in 3 M potassium thiocyanate for 5 min. The solution was then diluted to <1 M by PBS, and the supernatant was concentrated in a microconcentrator (Centricon 30; Amicon, Danvers, MA). Filters were used for several cycles of affinity purification.

Immunofluorescence and Immunoelectron Microscopy

For immunofluorescence microscopy, cells were grown on glass coverslips that in many experiments had been pretreated with 0.1% (wt/vol) poly-L-lysine solution (Sigma). The coverslips were thoroughly rinsed with 37°C warm PBS (pH 7.4), fixed for 5 min in methanol (at −20°C), treated with acetone for 30 s at −20°C, and air dried. After rehydration in PBS the slides were incubated with the primary antibodies for 30 min in a wet chamber, followed by three 5-min washes with PBS and incubation with Texas red–coupled or fluorescein-isothiocyanate–conjugated secondary antibodies (Dianova, Hamburg, FRG) for another 30 min. The secondary antibodies were usually pretreated for 5–10 min with 5% low fat milk powder and were centrifuged for 5 min. After three more 5-min washes with PBS, cells were dehydrated in ethanol, dried, and embedded in Elvanol. Fluorescence was detected either with a conventional fluorescence microscope (Axiophot; Carl Zeiss, Oberkochen, FRG) or a confocal laser scan microscope (LSM 310; Carl Zeiss) operating with a helium-neon laser at 543 nm and an argon ion laser at 488 nm.

Routine electron microscopy was as described (8, 9). For immunoelectron microscopy, several fixation procedures were tested. Best results were obtained when cells grown to near confluency on glass coverslips were washed three times with 37°C warm PBS and fixed in freshly prepared 2.5% or 3% formaldehyde in PBS for 30 min. Free aldehyde groups were quenched by incubation in 50 mM NH_4Cl in PBS for 2 × 5 min. Cells were lysed by a subsequent 10-min treatment with either 0.1% saponin in PBS or 0.05% digitonin in PBS. Incubation with the primary antibodies was for 30–60 min. After three washes with PBS, specimens were exposed for 2–4 h to secondary goat antibodies that had been coupled to gold particles (Amersham International). Cells were again washed three times with PBS and fixed for 20 min with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.4) containing 50 mM KCl and 2.5 mM $MgCl_2$. After several washes in 50 mM sodium cacodylate buffer (pH 7.4), cells were postfixed with 2% OsO_4 in cold buffer for 2 h, washed, dehydrated, flat-embedded in Epon, and sectioned as described (cf. 8, 9). In some experiments, the immunosignal was enhanced by silver amplification (Aurion; Bio Trend, Köln, FRG).

Subcellular Fractionation

Cells were washed with ice-cold PBS and lysed in cold (4°C) hypotonic buffer containing 10 mM triethanolamine-acetic acid (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.1 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride. The material was then homogenized either in a Potter-Elvejhem homogenizer by 25 up and down strokes or a tight-fitting Dounce homogenizer, and was subjected to centrifugation at 800 *g* for 5 min. The resulting supernatants (S1) were loaded on linear sucrose gradients that were centrifuged for 160 min at 270,000 *g* in a rotor (SW40 Ti; Beckman Instruments, Inc., Palo Alto, CA). Fractions were collected while recording the OD at 280 nm with a flow-through photometer, and the sucrose concentration of each fraction was determined with an Abbé refractometer. Alternatively, S1 supernatants were loaded on linear glycerol gradients (5–25%) with a sucrose cushion (50%) at the bottom, which were centrifuged for 60 min at 39,000 rpm in an SW40 rotor before collection of fractions. Aliquots were further analyzed either by the semiquantitative dot blot assay described by Wiedenmann et al. (55) or by an ELISA. For the ELISA, 10-μl aliquots were diluted with 90 μl PBS and pipetted into wells of a 96-well culture dish. After incubation under shaking for 1 h at room temperature or overnight at 4°C, the solution was removed, and the surface of the wells was blocked with 0.2% (wt/vol) BSA in PBS for 1 h. Bound proteins were reacted with synaptophysin antibodies (mAb SY38 or from rabbit sera) for 1 h, and the wells were washed several times with 0.05% (wt/vol) Tween 20 in PBS, followed by incubation with HRP-conjugated goat anti-mouse antibodies (Promega Biotec, Madison, WI) for 1 h. After several washes, HRP was detected with the chromogen 2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS; 50 μl/well of 1% [wt/vol] ABTS in reaction buffer [Boehringer Mannheim]), the reaction was stopped with 20 μl acetic acid, and absorbance was determined with a multichannel photometer at 405 nm. For direct immunoblot detection, proteins were precipitated from fractions by addition of 100% TCA to a final concentration of 15% and incubation at 4°C for 1–16 h. The precipitates were pelleted by centrifugation at 14,000 *g* for 10 min, washed with 90% acetone, 100% acetone, and dried. Resolubilization of the protein in buffer, separation by SDS-PAGE, immunoblotting, and detection of synaptophysin were done using standard procedures (cf. 26).

In some instances, synaptophysin-containing vesicles were further enriched from S1 sucrose gradient fractions or glycerol gradient fractions by immunoisolation (26). To this end, magnetic particles coated with sheep anti-mouse IgG1 (Dyna-Beads M-280; Dynal, Hamburg, FRG) were reacted with purified mAb SY38 (10 μg of HPLC-purified antibody for 100 μl of tightly packed beads) in blocking solution (PBS containing 10 mM EGTA, 0.1% BSA, 10 mg/ml phosphatidylcholine [Sigma]) for 20 min. Alternatively, in immunoisolation experiments using metabolically labeled cells, magnetic beads were first incubated at room temperature for 1 h with blocking solution supplemented with S1 supernatants from cells that do not express synaptophysin, and subsequently reacted with SY38 hybridoma supernatant in blocking solution for 1 h. After several washes with blocking solution, beads were added to selected cellular fractions and were incubated at room temperature between 20 min and 1 h. Several washes with PBS containing 10 mM EGTA, and for the most stringent washing step, 1 M KCl, followed (see also 26). Immunoisolated material was either pelleted by centrifugation for 2 min at 13,000 *g* and solubilized in SDS-PAGE buffer for subsequent immunoblot analysis and autoradiography, or it was centrifuged for 5 min at 800 *g*, followed by fixation in buffer containing 2.5% glutaraldehyde (see above), treatment with buffered 2% OsO_4, dehydration, embedding in Epon, ultrathin sectioning, and visualization under a Siemens 101 electron microscope (Berlin, FRG).

Immunoprecipitation from S1 (800 *g* supernatant of lysed and homogenized cells) was done with Eupergit C1Z beads (Röhm, Weiterstadt, FRG), to which rabbit or guinea pig antibodies against synaptophysin had been coupled (for methodological details see 10).

Endoglycosidase Treatment

In some instances, immunoprecipitated synaptophysin was treated with either endoglycosidase H (Boehringer Mannheim) after solubilization (6 min at 95°C) in 0.66% SDS, 33 mM sodiumphosphate (pH 5.4) for 20 h at 37°C, or with endoglycosidase F (Boehringer Mannheim) after solubilization (6 min at 95°C) in 0.1 M sodium phosphate, pH 8.6, 1.2% NP-40, 0.2% SDS, 10 mM EDTA, 1 mM PMSF, 140 mM β-mercaptoethanol, and subsequent dilution with H_2O (1:3) for 20 h at 37°C.

Results

Separation of Synaptophysin-containing Vesicles from Other Vesicles

We have previously described sublines of stably transfected human PLC cells that constitutively express the transfected

rat synaptophysin gene (26). To study the distribution of the gene product in non-NE cells in greater detail, we tested a variety of gene constructs and selection protocols. Clone PLC-6S4, which was selected for puromycin resistance (4 μg/ml) after transfection with pSR[10] (see Materials and Methods), was used for most of the experiments described here, as it showed the highest level of expression determined by Northern blot hybridization and immunoblot quantitation. The resulting synaptophysin steady-state level was approximately one fifth of that determined in our cultures of PC12 cells (9).

In the PLC-6S4 cells, as well as in the other transfected cell lines examined, the vast majority of the synaptophysin was in an endoglycosidase H-resistant form (Fig. 1), and it appeared mostly in the multipunctate pattern of scattered vesicles (cf. 26). Under normal steady-state conditions, we did not detect appreciable amounts of immature, i.e., endoglycosidase H-sensitive synaptophysin, and we also did not see any synaptophysin in the ER. The half life of the mature synaptophysin in PLC-6S4 cells under our growth conditions exceeded 12 h, as determined by pulse chase experiments (not shown).

Fractionation of the total cellular vesicles by glycerol gradient centrifugation, followed by ELISA with synaptophysin mAb SY38 (Fig. 2 *A*), showed that synaptophysin was contained in a major vesicle type with a distinct sedimentation characteristic but it appeared over a range of fractions, showing some enrichment in a "light peak" fraction (Fig. 2 *A*, fractions 6–12) and at the density cushion. When these fractions were further examined by immunoisolation experiments using mAb SY38, it was found that synaptophysin-containing vesicle fractions also contained the transferrin receptor as a marker for endocytosis, although in variable amounts (Fig. 2, *B* and *B'*).

To examine the possible inclusion of synaptophysin in vesicles of the constitutive secretory pathway (cf 22, 23, 38) of PLC cells, we compared its distribution with that of the hepatitis B surface antigen (HBs), a characteristic integral membrane protein of secretory vesicles of this cell line (30). Using sucrose gradient centrifugation (Fig. 2 *C*), vesicles containing synaptophysin could be clearly separated from vesicles carrying HBs, showing that the constitutive pathway secretory vesicles differ drastically in size and sedimentation behavior from the majority of the synaptophysin vesicles. This observed steady state distribution does not, of course, rule out the possibility that some synaptophysin molecules exist as transient passengers in constitutive pathway vesicles.

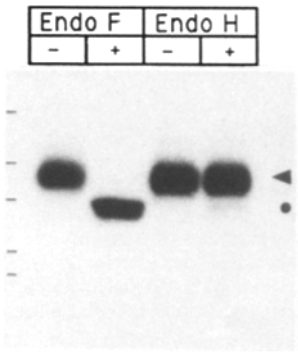


Figure 1. Autoradiograph of SDS-PAGE (15%) showing radioactively labeled synaptophysin that was immunoprecipitated from human hepatoma PLC-6S4 cells with rabbit antibodies to synaptophysin and treated for 20 h at 37°C with 0.2 U endoglycosidase F (lane *2*, untreated control in lane *1*) or endoglycosidase H (lane *4*, untreated control in lane *3*). Cell proteins were metabolically labeled by overnight incubation of ~10^6 cells with 150 μCi [^{35}S]methionine (cf. 26). Bars on the left margin denote the relative positions of coelectrophoresed reference proteins (*top–bottom*: BSA, M_r ~67,000; ovalbumin, M_r ~45,000; glyceraldehyde-2-phosphate dehydrogenase, M_r ~36,000; carbonic anhydrase, M_r ~29,000; trypsinogen, M_r ~24,000). The position of the glycosylated form of synaptophysin is indicated by an *arrowhead*, that of the aglycon obtained by endoglycosidase F by a dot.

Several studies have shown that in NE cells, as well as in transfected non-NE cells, synaptophysin reaches the plasma membrane, where it can be directly visualized (26) and biochemically labeled, followed by clustering and endocytosis (e.g., 2, 3, 19, 26, 28, 38). Using the fluid-phase marker HRP, we have previously shown in PLC cells that only a subfraction of the HRP-labeled vesicles was also positive for synaptophysin (26). This was also found throughout the present study, and it was corroborated by biochemical experiments. When, for example, PLC-6S4 cells were incubated with HRP for ≤2 h, following the endocytosis protocol described by Johnston et al. (19) for CHO cells, and vesicles were fractionated by sucrose gradient centrifugation (Fig. 2 *D*), the majority of HRP was not detected in fractions containing synaptophysin (for a minor synaptophysin-containing fraction see Fig. 2 *D*, fraction 11). HRP was practically absent from the fractions containing the small synaptophysin-rich vesicles (Fig. 2 *D*, fractions 3–6). Similar results were obtained after shorter incubation times (cf. 26 and data not shown).

When we labeled the surface-bound TFR of PLC-6S4 cells, allowed for endocytosis, and analyzed the distribution of labeled vesicles, the low resolution power of our sucrose gradient analyses did not allow the separation of TFR and synaptophysin vesicles under the conditions chosen (Fig. 2 *D*). Subsequent immunoisolation with synaptophysin antibodies, however, resulted in the enrichment of vesicles that were rich in synaptophysin, but did not contain amounts of TFR detectable under these conditions (Fig. 2 *E*, lanes *3* and *3'*).

Further analysis of the immunoisolates by electron microscopy showed that the bead-attached, synaptophysin-rich vesicles were mostly small (<100 nm diameter), with an electron-translucent interior (Fig. 3), similar to those detectable in situ (see below). These observations indicated that a sizeable portion of the TFR and the synaptophysin is contained in different vesicles and that a subtype of synaptophysin-rich SET vesicles contains very little, if any TFR.

Light and Electron Microscopic Identification of Synaptophysin-containing Vesicles

To compare directly the overall distribution of synaptophysin with that of TFR-containing vesicles, we performed double-label immunofluorescence microscopy.

Drastic differences in the distribution of the membrane proteins studied were observed (Figs. 4, *a* and *a'*, and 5, *a* and *a'*). Most of the TFR immunoreactivity, detected either by mAb B3/25 or mAb OKT9, was seen as a coarsely granular staining, often showing enrichment in a juxtanuclear aggregate, whereas synaptophysin appeared in the typical finely punctate pattern throughout most of the cytoplasm. Small granular structures coincidentally positive for both TFR and synaptophysin were also repeatedly noticed (for a detailed comparison see inserts in Fig. 5, *a* and *b*).

A similar differential localization was seen when the distribution of synaptophysin was compared with that of the

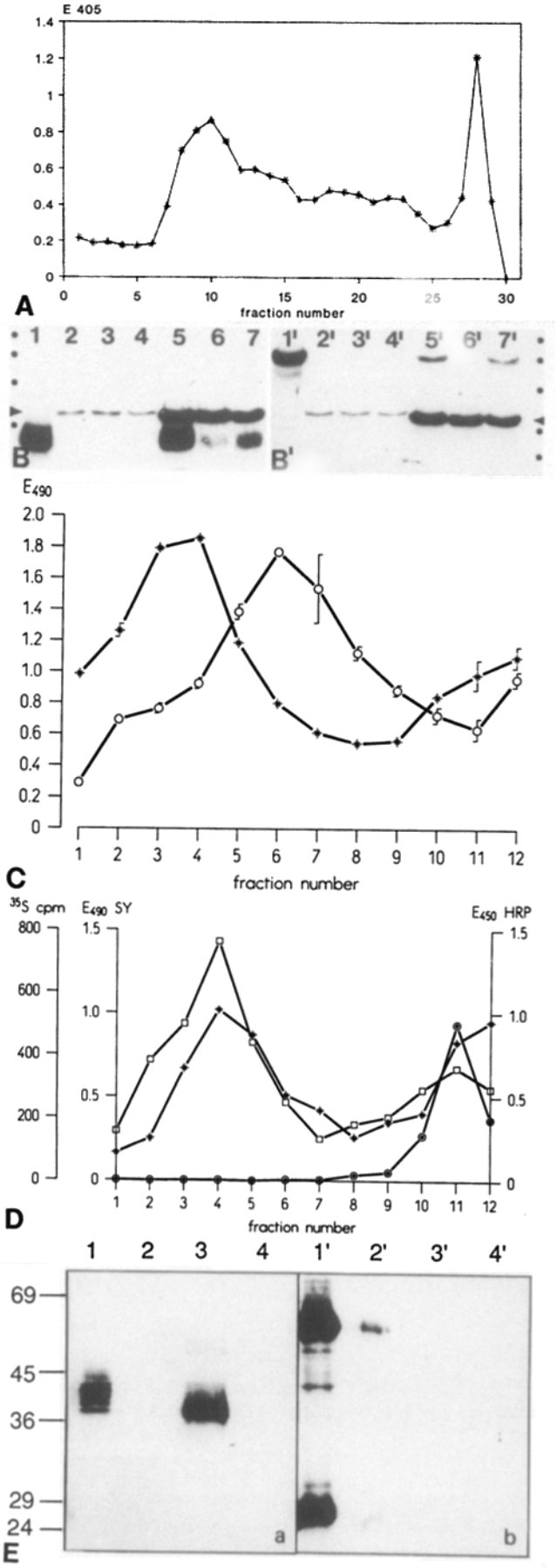


Figure 2. Characterization of synaptophysin-containing vesicles from PLC-6S4 cells by centrifugation and immunoisolation techniques. (*A*) Quantitation of relative synaptophysin concentration in glycerol gradient fractions. 500 μl of a postnuclear (1,000 *g*) supernatant (2 mg protein/1 ml) were loaded onto a linear 5–25% glycerol gradient with a 50% sucrose cushion at the bottom. After centrifugation at 39,000 rpm for 60 min in a SW40 Ti rotor (Beckman) 400 μl fractions were collected from top (fraction 1) to bottom. The relative synaptophysin concentration was determined with an ELISA (E, absorbance at 405 nm in OD units is given on the ordinate). (*B*) Immunoblots of immunoisolates from vesicle fractions of postnuclear supernatant. Polypeptides contained in equal volumes of immunoisolates were separated by SDS-PAGE (12%), transferred to nitrocellulose filters, and reacted with beads coated with mAb SY38, using the enhanced chemiluminescence system (Amersham) for detection (see Materials and Methods). As positive control, lane *1* contains total postnuclear supernatant (20 μg). For negative controls, magnetic beads coated only with secondary antibodies were incubated with postnuclear supernatant (lane *2*), pooled glycerol gradient fractions 6–12 (lane *3*) or 25–30 (lane *4*). Synaptophysin-containing vesicles were isolated with mAb SY38 from postnuclear supernatant (lane *5*), in comparison with pooled glycerol gradient fractions 6–12 (lane *6*) or 25–30 (lane *7*). The relative position of molecular mass standards are indicated by dots (from top to bottom: β-galactosidase, M_r ~116,000; phosphorylase b, M_r ~97,400; BSA, M_r ~67,000; ovalbumin, M_r ~45,000). The arrowhead denotes the position of the immunoglobulin heavy chain detected by the secondary antibody. Note synaptophysin reaction in lanes *1* and *5–7*. (*B'*) Immunoblot of the same fractions as in *B*, but reacted with mAb OKT9 against human TFR. Same experimental conditions and denotations as in *B*. Note markedly positive reaction in lanes *1'*, *5'*, and *7'*. (*C*) Quantitation of immune reactions of blotted protein fractions (fraction numbers) obtained after velocity gradient centrifugation (5–30% linear sucrose gradient; 260,000 *g* for 160 min) of overlayed postnuclear (1,000 *g*) supernatants. Protein fractions collected from bottom (*left*) to top (*right*) were either reacted with mAb SY38 against synaptophysin (*asterisks*) or with antibodies against the bulk flow marker HBs (*circles*). Ordinate presents mean values of extinction at 490 nm from seven independent experiments. Synaptophysin peak fractions 3 and 4 were 20% and 19% with respect to sucrose concentration, whereas the HBs-containing peak fraction 6 contained 16% sucrose. (*D*) Graphs showing the distribution of synaptophysin-containing vesicles in comparison to those containing either TFR (*open squares*) or HRP (*dotted circles*) in sucrose gradient fractions from PLC-6S4 cells (same procedures as in *C*). Synaptophysin immunoreactivity (*asterisks*) was determined by quantitation of absorption at 490 nm in a dot blot immunoassay using mAb SY38. For detection of TFR, cells had been incubated with TFR mAb B3/25 (10 μg/ml), together with secondary ^{35}S-labeled sheep anti-mouse Ig (20 μCi/10-cm culture dish) for 1 h before harvesting. Radioactivity was measured in 50-μl aliquots of sucrose gradient fractions by liquid scintillation counting. For uptake of HRP, cells were incubated for 2 h with HRP (10 mg/ml) and peroxidase activity was determined in 0.2-ml aliquots of sucrose gradient fractions by adsorption at 450 nm 30 min after reaction of *o*-dianisidine in the presence of H_2O_2. Similar results were obtained with TFR mAb OKT9. (*E*) Immunoblot using rabbit synaptophysin antibodies and the alkaline phosphatase detection system (*a*) and corresponding autoradiograph (*b*) of polypeptides separated by SDS-PAGE (15%) from cells labeled with TFR antibodies (see *D*), demonstrating the segregation of synaptophysin- and TFR-containing vesicles. 5% of postnuclear supernatant was loaded in lanes *1* and *1'*, 1% of pooled sucrose fractions 4–6 were loaded in lanes *2* and *2'*. Synaptophysin-containing vesicles were further enriched from 50% of this pool by immunoisolation using magnetic beads coated with mAb SY38 via secondary antibodies (lanes *3* and *3'*; see Materials and Methods). As a negative control, the rest of the sucrose gradient pool fraction was incubated with magnetic beads coated only with secondary antibodies (lanes *4* and *4'*). The relative position and relative molecular mass values (in kilodaltons) of coelectrophoresed molecular mass references (cf. Fig. 1) are indicated at the left margin. Note the enrichment of synaptophysin and concomitant reduction of the ^{35}S-labeled heavy and light chains of the secondary antibody associated and endocytosed with the TFR in the immunoisolated vesicles. Note that the strong synaptophysin signal in lane *3* does not correspond to a TFR reaction (lane *3'*). Only after extended exposure a very weak signal could be detected in lane *3'* (not shown).

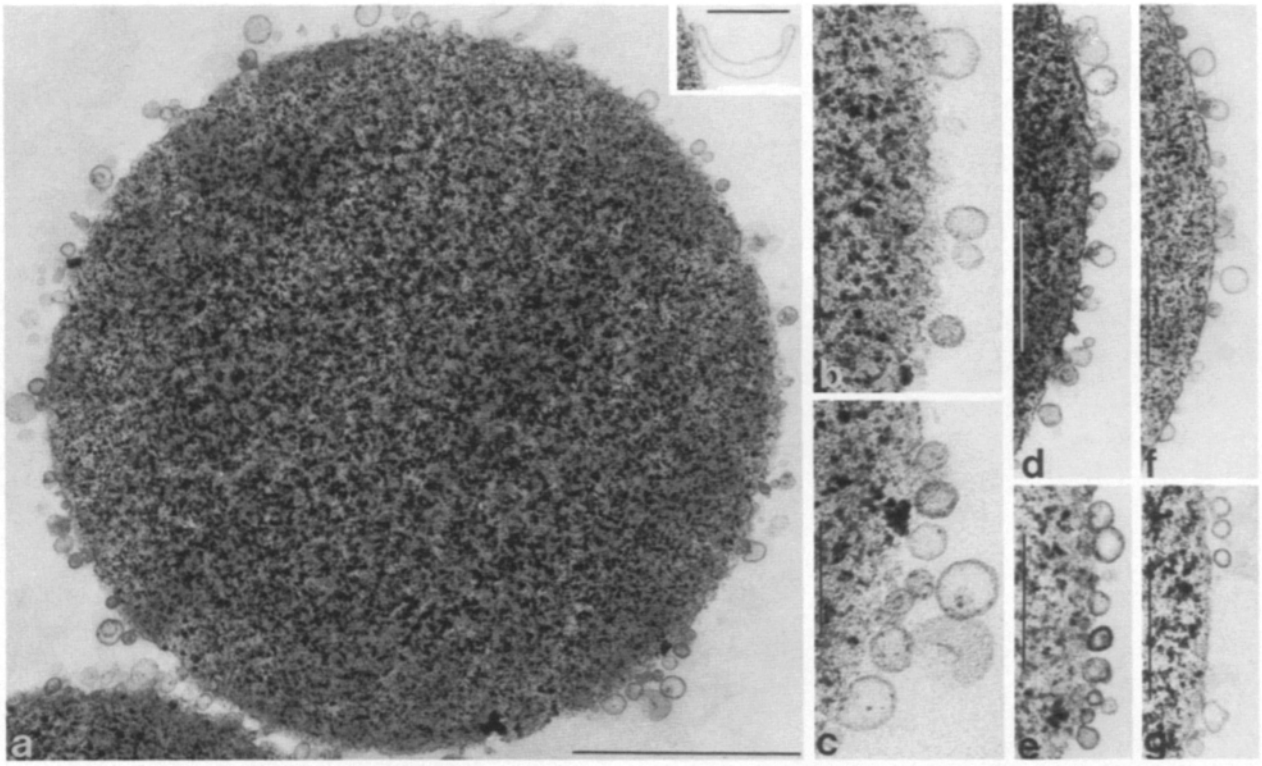


Figure 3. Electron micrographs of immunoisolated vesicles from PLC cells transfected with the synaptophysin gene construct pSR[7] (cf. 26). Synaptophysin-containing vesicles were immunoadsorbed to magnetic beads coated with mAb SY38. The micrograph in (*a*) shows one such magnetic bead (diameter ~3.2 μm) surrounded by a number of tightly attached, rather small vesicles (most diameters = 30–100 nm). The inset in *a* depicts a tubular structure that was seen attached to another bead. (*b* and *c*) Details of *a* at higher magnification. (*d–g*) Demonstration of the abundance and size variation of SET vesicles adsorbed to other beads. Bars, 1 μm in *a*, 500 nm in inset of *a* and *d–g*; 200 nm in *b* and *c*.

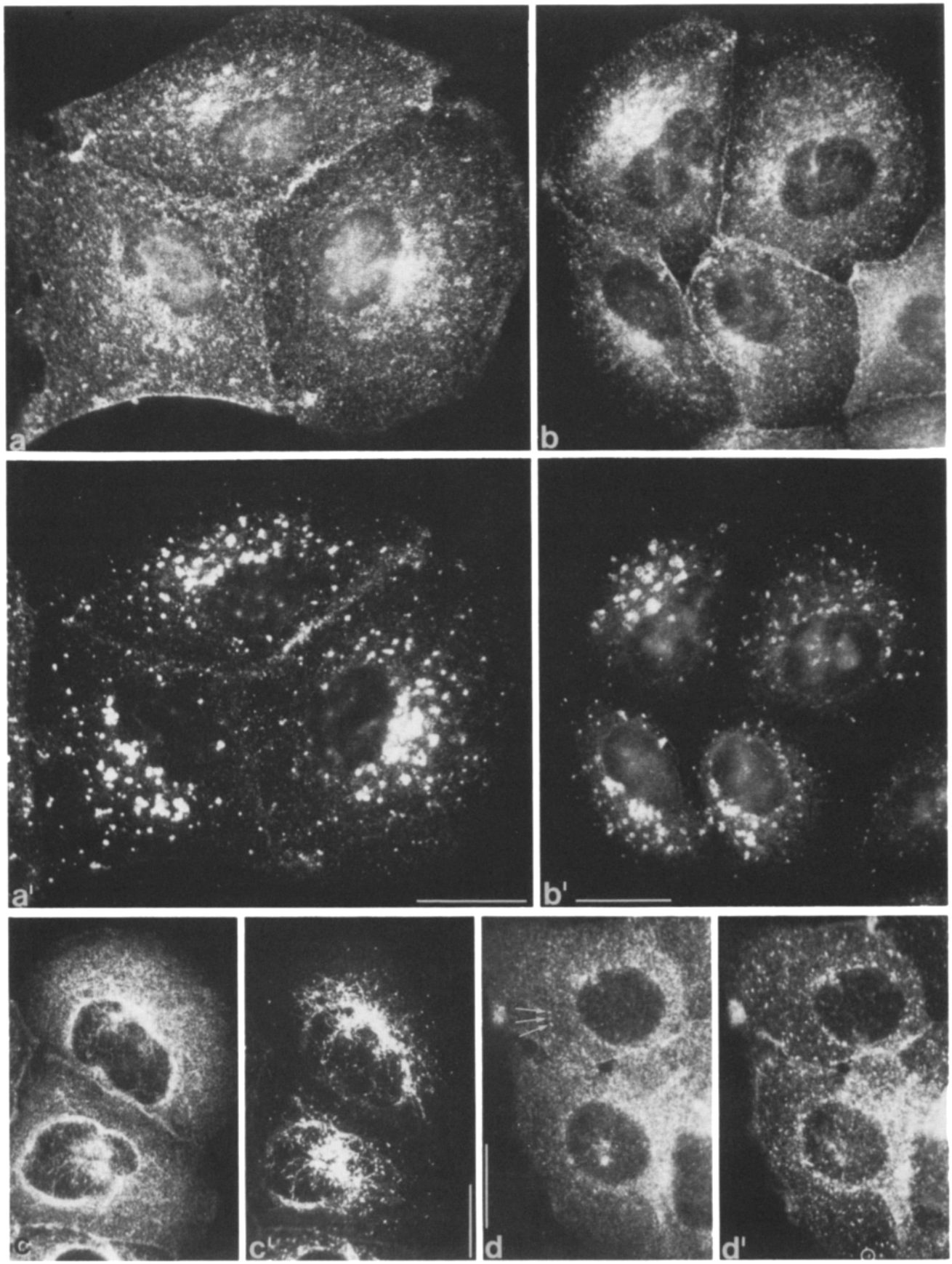
TFR-containing vesicles, visualized by bound and endocytosed ligand at subsaturation levels (Fig. 4, *b* and *b'*). Using various drugs, including brefeldin A, that can alter the distribution of endosomal structures and markers (cf. 47), such as TFR, we did see some but by no means a complete colocalization of TFR and synaptophysin (Fig. 4, *c* and *c'*).

When such colocalization experiments were performed on various other transiently transfected epithelial cells, similar results were obtained. As shown for the example of A-431 cells (Fig. 4, *d* and *d'*), a number of "dots" were fluorescent for both antigens, but many others showed mutual exclusivity for either synaptophysin or TFR.

To identify and characterize the synaptophysin-containing structures appearing as small "fluorescent dots" in immunofluorescence microscopy (e.g., see Fig. 5 *a*), we performed immunoelectron microscopy on such cells using the same primary antibodies and secondary immunogold labeling (Fig. 5 *c*). The majority of the structures decorated by synaptophysin antibodies, as visualized by gold particles, were SET vesicles with diameters in the 30–90-nm range (Fig. 5 *c*, *arrows*), whereas minor proportions of somewhat larger vesicles were also labeled, including occasional late endosomes appearing as multivesicular bodies (see below). In quantitative evaluations in the form of histograms (not shown), 83% of the immunogold-labeled vesicles fell into the diameter size classes of 60–90 nm (43%) and <60 nm (40%), i.e., the size category defined as SLMV. For comparison, the corresponding figures for synaptophysin antibody–labeled vesicles in the "typical" NE cells of line PC12 were 92% (30–60 nm) and 6% (60–90 nm).

To visualize directly the individual vesicles of the three different kinds (SY^+/TFR^-, SY^-/TRF^+, SY^+/TFR^+), laser

Figure 4. Double-label immunolocalization of synaptophysin (*a–d*) with TFR, visualized by TFR antibodies (*a'*, *c'*, *d'*) or by bound and endocytosed transferrin (*b'*), in methanol/acetone-fixed PLC-6S4 (*a–c* and *a'–c'*) or A431-5S4 (*d* and *d'*) cells. (*a* and *a'*) Synaptophysin was detected with affinity-purified rabbit antibodies, TFR with murine mAb B3/25. (*b* and *b'*) Colocalization of synaptophysin (using mAb SY38) and transferrin using affinity-purified rabbit antibodies. Transferrin was added to cells before fixation at a concentration of 15 μg/ml for 1 h. (*c* and *c'*) Cells were incubated with 20 μg/ml cycloheximide for 4 h and with 5 μg/ml brefeldin A and 10 μg/ml TFR antibody B3/25 for 3 h before fixation. Endocytosed TFR antibody was detected only with labeled secondary antibody, whereas affinity-purified rabbit antibodies were used for detection of synaptophysin. (*d* and *d'*) Colocalization of synaptophysin and TFR in the synaptophysin-producing selected A-431 cell subline 5S4, using the same reagents as in (*a* and *a'*). Arrows in *d* point to a series of fluorescent dots not seen in *d'*, whereas the circle in *d* marks one of the dots not present in *d*. Note the differences in overall distribution between both vesicle populations in all pairs of micrographs. Bars, 20 μm.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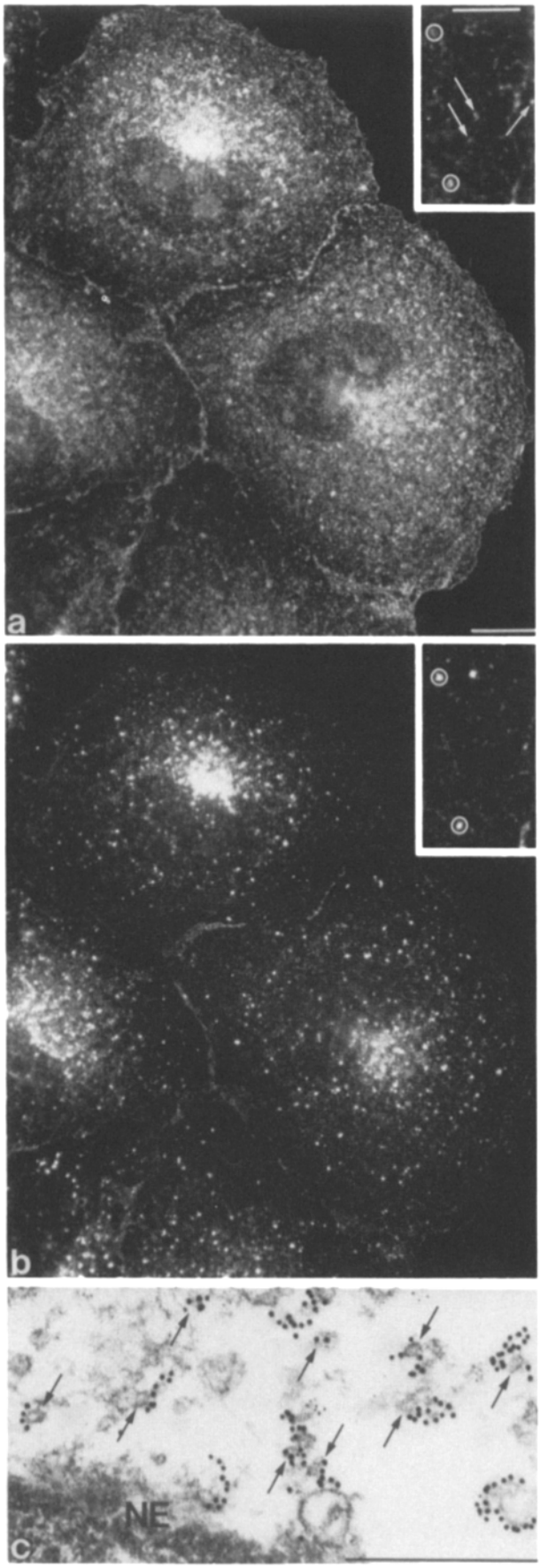

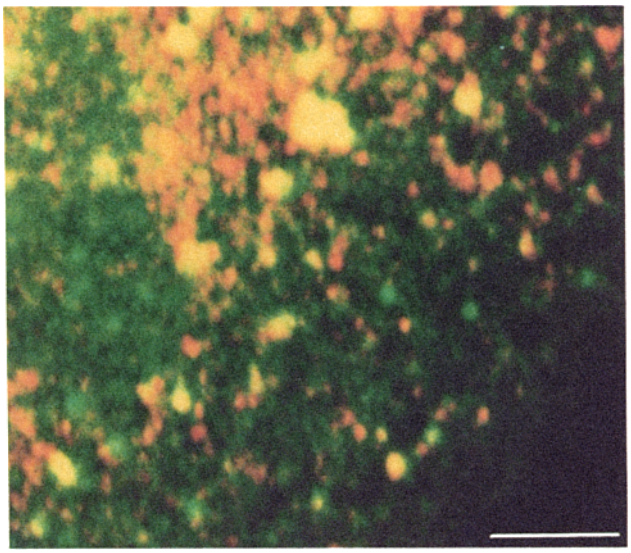

Figure 6. Photomicrograph showing simultaneous immunocolocalization of synaptophysin (*green*) and the TFR (*red*) in PLC-6S4 cells. Methanol/acetone-fixed cells were incubated simultaneously with affinity-purified synaptophysin antibodies from rabbit and mAb B3/25 against TFR, followed by reaction with preadsorbed secondary fluorescein-conjugated goat anti-rabbit IgG antibodies and Texas red-coupled goat anti-mouse IgG antibodies. Fluorescence was recorded by simultaneous scanning in the same focal plane by a helium-neon and argon laser. Note the presence of orange/red and green punctate staining in addition to yellowish dots, indicating separate (*orange/red*, and *green*) localization or codistribution (*yellow*; the number of codistributive sites may be somewhat overestimated because of the limitations of resolution of closely adjacent vesicles). Bar, 5 μm.

scan microscopy and digital imaging procedures were used (Fig. 6). Optimal resolution was usually obtained in the peripheral cytoplasm, where the cells were flattened out. In general, synaptophysin immunoreactivity (green color) was more prominent than TFR immunofluorescence (orange/red). In addition, yellow fluorescent "dots" were regularly seen, representing vesicles containing both antigens.

The numerous sites of differential synaptophysin and TFR signals in punctate immunofluorescent signals and electron microscopic vesicles, respectively, show that both membrane proteins can greatly differ in their enrichment in a specific vesicle, although the presence of a few molecules in

Figure 5. Details of immunolocalization of synaptophysin and TFR in PLC-6S4 cells. (*a* and *b*) Double-label immunofluorescence microscopy of synaptophysin using affinity-purified antibodies from rabbit (*a*), and TFR using mAb OKT9 (*b*) on methanol/acetone-fixed cells. Note the only partially coincident, but mostly different distribution of both antigens. The insets show a selected region at higher magnification, demonstrating examples of colocalization (*circles*), as well as structures labeled only by synpatophysin antibodies (*a*, *arrows*). (*c*) Representative immunoelectron micrograph showing the synaptophysin-containing vesicles. Cells were fixed with 2.5% formaldehyde and lysed with 0.1% saponin before antibody incubation. Note immunogold localization predominantly in small (diameter = 30–90 nm), electron-translucent vesicles (*arrows*). *NE*, nuclear, envelope. Bar, 0.5 μm.

the "negative" vesicles cannot be rigorously excluded, considering the limits of detection of the specific methods.

Immunoelectron Microscopic Comparison of Synaptophysin and Endocytosed TFR

To exclude a preferential intravesicular location of the TFR epitope, resulting in the shielding from detectability by inaccessibility, we also used a protocol in which the surface-bound TFR of living cells were labeled with TFR antibodies and then with the corresponding labeled secondary antibodies. The distribution of the endocytosed TFR-antibody complexes was indistinguishable from that of the receptor (Figs. 4–6; for use of labeled primary antibody, e.g., see references 15, 16). Fig. 7 presents the characteristic distribution of endocytosed TFR in transfected PLC cells, which clearly differs from that of synaptophysin (compare, e.g., Fig. 5 *c*).

At early time points, immunogold particles were mostly detected in clathrin-coated pits or in vesicles close to the plasma membrane, including certain elongated vesicles, which possibly correspond to tubular and "cup-shaped" early endosomes (15, 16, 47). TFR label could be seen in vesicles of different sizes and morphological appearances. At later time points, gold particles were contained in small (50–150 nm) cortical vesicles or tubular structures. Similarly sized and labeled vesicles were also found in the *trans*-Golgi network and other parts of the Golgi region (cf. 12, 18, 56). In addition, TFR immunoreactivity was found within larger vesicles corresponding to late endosomes or structures resembling multivesicular bodies (13, 16, 36, 47).

In double-label immunoelectron microscopy, we examined with particular care areas in which label for both markers could be seen in the same field (e.g., Fig. 8, *a–f*). Analysis of sections through numerous cells showed the simultaneous presence of three major kinds of labeled vesicles.

(*a*) Corresponding to the immunofluorescence pattern (e.g., Figs. 4, *a'* and *b*, and 5 *b*), TFR antibodies reacted with certain vesicles of rather variable sizes, including many large ones such as "multivesicular bodies," but also with smaller vesicles and some tubular extensions, in the absence of any detectable synaptophysin label.

(*b*) Conversely, a large number of smaller SET vesicles were only labeled by antibodies to synaptophysin, but not with TFR antibodies. These vesicles also differed in size, and we grouped them into those with larger (>90 nm) and those with smaller (30–90 nm) diameter. The latter category corresponds to the SLMV of PC12 and other NE cells (e.g., see above and references 5, 9, 29, 33, 37, 38, 45, 52, 54). Occasionally, we observed some elongated membrane-bound structures suggestive of "tubular extensions" that were also positive for synaptophysin (for example, see Fig. 8 *a*), similar to the "cisternal" or "tubular" structures found positive for synaptophysin in pancreatic islet β cells (37).

(*c*) A subtype of variously sized vesicles was positive for both internalized TFR and synaptophysin antibodies on the cytoplasmic surface of the vesicle.

Similar results of immunofluorescence microscopy and single- or double-label immunogold electron microscopy were obtained for selected subclones of transfected cells of the hepatoma-derived cell line SK-Hep-1 (subclone 1S4) and the vulvar carcinoma-derived cell line A-431 clone E_3 (subclone 5S4). In all transfected cell clones, the induced expression of the gene encoding rat synaptophysin and the inclusion of the product in SET was a stable feature, observed in the oldest PLC subline, now for >4 yr of continued cell proliferation.

Discussion

Synaptophysin, a major constituent glycoprotein of small (30–90 nm) electron translucent vesicles, i.e., PSV in neurons and SLMV in diverse NE cells, has become a model molecule for studying mechanisms of specific membrane protein sorting and vesicle formation. It is generally agreed that in the presynaptic cytoplasm of neurons synaptophysin, together with other PSV proteins, can go through several cycles of triggered exocytosis and endocytotic recycling, probably via coated vesicles (cf. 14, 32, 53). Controversy, however, starts when it comes to the questions of the site of initial assembly and sorting of synaptophysin and other vesicular components, and of the form and the route by which these vesicle proteins reach the presynaptic region (e.g., 28, 31, 38, 46).

Even more controversial are the discussions on the molecular principles and pathways of the formation of synaptophysin-containing vesicles in NE cells, including the PC12 cell line, and in non-NE cells forced to synthesize synaptophysin by transfection with recombinant gene constructs (2–4, 9, 19, 26, 28, 29, 38). We (26) and others (19, 28) have reported that in such transfected non-NE cells, most of the synaptophysin accumulates in cytoplasmic vesicles, including those of the SLMV size class. However, comparing transfected cell cultures of fibroblastoid lines (CHO and 3T3) with NE cells (PC12), Kelly and colleagues (3, 21–23, 28, see also 2, 5, 6) have stated that synaptophysin is sorted into SET vesicles of the "correct" (PSV or SLMV) size class only in "competent," i.e., NE cells, whereas non-NE cells would be unable to sort this molecule into such vesicles, resulting in their entrance into the lysosomal pathway and/or their unsorted inclusion, together with diverse plasma membrane molecules in the general endosomal-plasma membrane recycling shuttle (see also 5). These authors, as well as Cameron et al. (2), concluded therefore that the sorting machinery for PSV and SLMV is only present in neurons and NE cells (for reviews see 21, 22).

In contrast, using human epithelial PLC cells, we have reported that most of the foreign synaptophysin molecules accumulate in a certain type of SET vesicle in which they can represent, under certain conditions, a predominant protein (26). Our present study in three different epithelial cell lines confirms and extends this observation by the demonstration, with three independent methods (particle fractionation, immunoisolation, and immunolocalization), of synaptophysin-containing SET vesicles of diameters in the range of 30–90 nm, as it is characteristic of SLMV of typical NE cells (5, 9, 29, 33, 37, 45). We take this specific enrichment of synaptophysin in a distinct type of SET vesicle, which is morphologically indistinguishable from SLMV, to indicate the existence of a sorting mechanism functioning for a NE-typical molecule even in a non-NE cell.

Our conclusion of the ability of various non-NE cells to direct "foreign" synaptophysin to ST vesicles with the correct size for SLMV finds also support in published data, though not the conclusions of other authors. Careful reexamination of the micrographs of the groups studying fibroblastoid cells

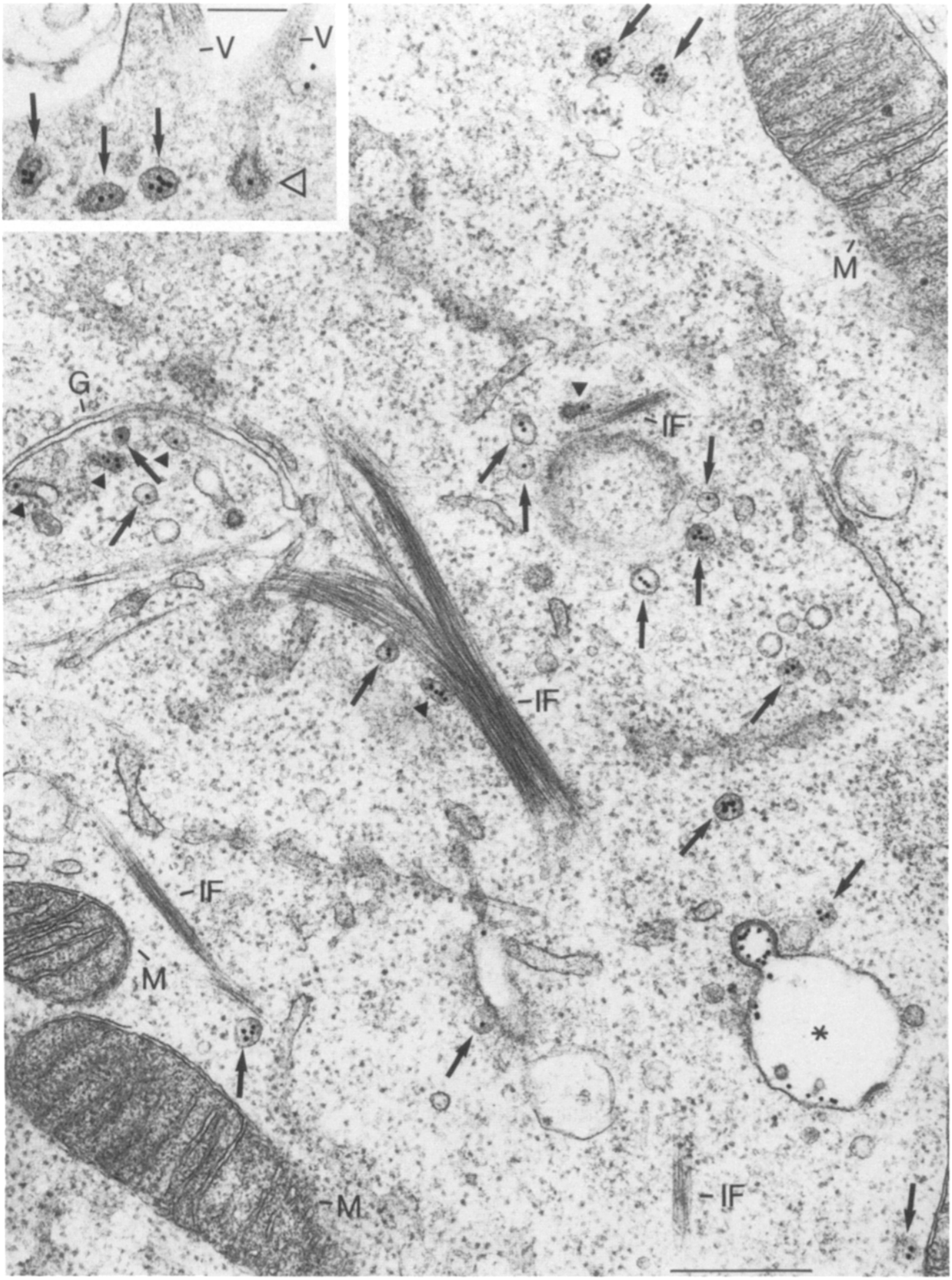

Figure 7. Immunoelectron microscopic localization of endocytosed TFR in PLC-6S4 cells. Iron-depleted cells were incubated with TFR mAb B3/25 (10 μg/ml) and secondary antibodies coupled to 15-nm colloidal gold particles (diluted 2:3 in DME) at 4°C for 30 min, and endocytotic compartments were saturated by further incubation at 37°C for 1 h, followed by fixation in buffer containing 3% formaldehyde (see Materials and Methods). Gold particles can be seen in vesicles of widely varying sizes (*arrows*), some of which show tubular extensions (*arrowheads*) reminiscent of the endosomal network. Other labeled vesicles are next to Golgi cisternae (*G*). In addition, many gold particles are contained in late endosomes and structures resembling multivesicular bodies (*asterisks*; we have not determined whether these "vesicle-

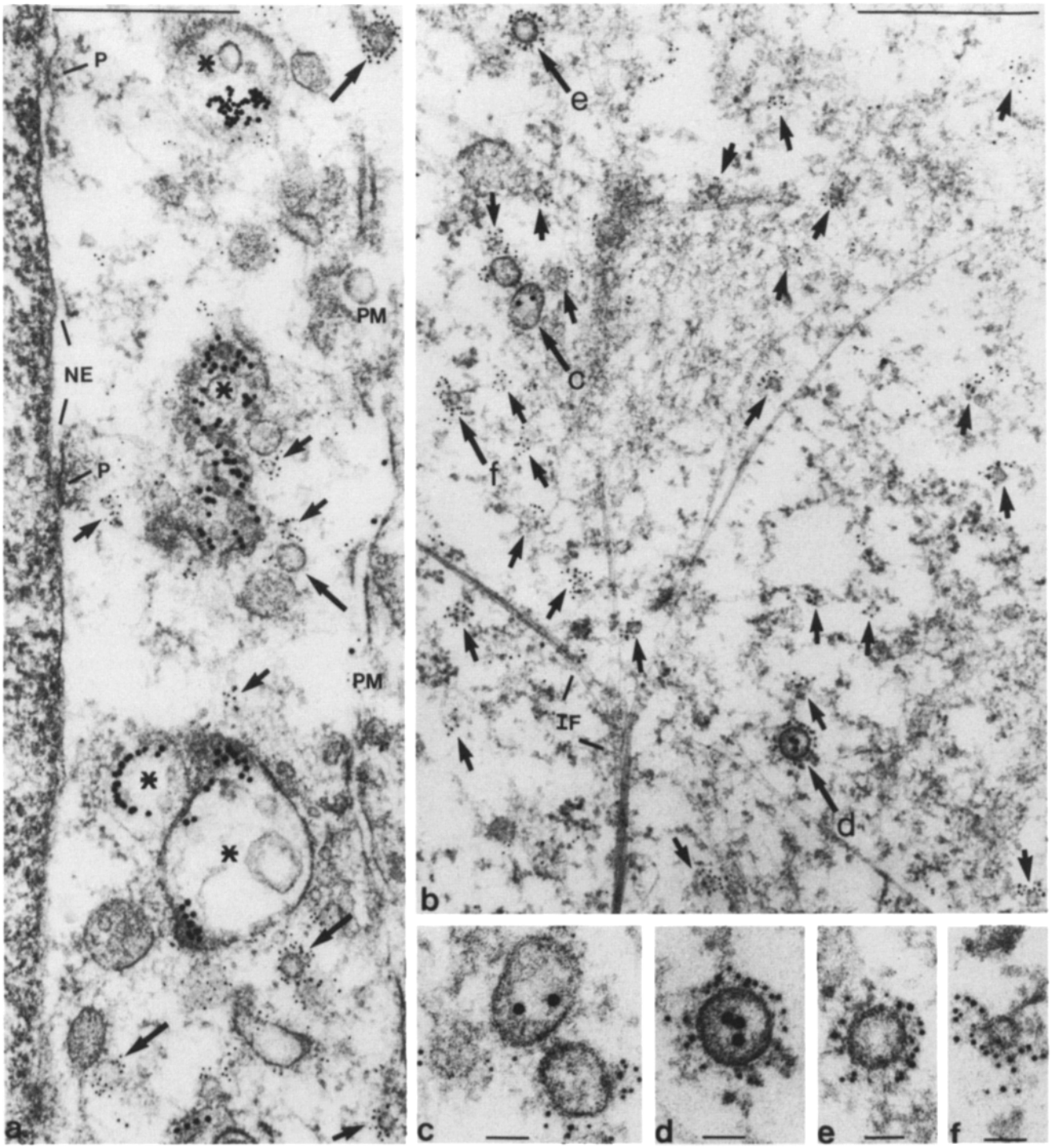

Figure 8. Double-label immunoelectron microscopy of PLC-6S4 cells, detecting TFR by mAb B3/25 and 15-nm gold-conjugated secondary antibodies before fixation with 3% formaldehyde in PBS (as in Fig. 7) and partial cell lysis with 0.1% saponin, followed by incubation with affinity-purified synaptophysin antibodies (from rabbit) and secondary antibodies coupled to 5-nm gold particles. (*a*) Juxtanuclear region (*NE*, nuclear envelope) and cytoplasm (*PM*, plasma membrane), showing that the TFR-specific 15-nm gold particles are predominantly detected in the lumina of large vesicles, including those resembling multivesicular bodies (*asterisks*), whereas small gold particles decorate the outside of synaptophysin-containing vesicles of larger (90–120 nm; *long arrows*) and smaller (30–90 nm; some are denoted by small arrows) diameter. (*b*) Survey micrograph of a large cytoplasmic area showing the abundance of small (<120 nm) vesicles decorated by 5-nm gold particles, i.e., containing synaptophysin, and somewhat larger (90–150 nm) vesicles that are positive for either synaptophysin (5-nm gold; one example is labeled *e*), or only for internalized TFR (15-nm gold; *c*), or for both (for example see *d*). These different types of vesicle are shown at higher magnification in *c–e*; for comparison, an example of the small (≤60 nm) vesicles is shown in (*f*). Bars, 0.5 μm in *a* and *b*; 50 nm in *c–f*.

containing vesicles" contain lysosomal activities or represent "receptosomes" *sensu* [36]). *M*, mitochondria; *IF*, intermediate-sized filaments. The inset (*upper left*) shows the distribution of TFR in cells that were only incubated for 5 min at 37°C before fixation. The region shown is in close proximity to the plasma membrane and contains gold label within early endosomes (arrows denote membrane-bound structures, open arrowheads denote a clathrin-coated pit budding off the plasma membrane). *V*, villus. Bars, 0.5 μm, 0.2 μm in inset.

transfected to produce synaptophysin has shown that indeed in the synaptophysin vesicle populations described vesicles of the typical SLMV size category were predominant, with some size heterogeneity. For example, the immunoelectron microscopy of Feany et al. (see, e.g., Fig. 5 *B* in reference 6) shows that of eight identifiable vesicles shown to represent a "relatively uniform vesicle population with an 80-nm diameter," six (75%) fall into the size class of 60-nm diameter and below, whereas two were much larger, falling into the ≥100 nm class. Correspondingly, the majority of the synaptophysin-containing "microvesicles" described in the CHO cells transfected by Johnston et al. (19) and Cameron et al. (2) have typical SLMV diameters of 90 nm and below (see Figs. 5 and 9 in reference 2 and Figs. 3–5 in reference 19), so that the authors concluded that the synaptophysin vesicles in the transfected CHO cells are similar to the genuine SLMV present in NE cells, such as the PC12 line.

However, in our transfected cells, not all synaptophysin-containing SET vesicles are identical and not all the synaptophysin synthesized is distributed in the same way. In the transfected epithelial cells, we have also found, in three different epithelial cell lines, that some of the synaptophysin occurs in endocytotically formed vesicles, resulting from recycling through the endosome compartment, as shown by the colocalization with TFR (26 and the present study). Other authors (3, 6, 19, 21–23, 28) have interpreted their observations in transfected fibroblastoid cells to indicate that all synaptophysin-containing vesicles are exclusively endosomes and represent nonsorted admixtures with various recycling plasma membrane markers, such as receptors for transferrin and LDL. However, this generalization implicating uniformity, i.e., only one vesicle route and thus only one type of cytoplasmic steady state vesicle, does not seem justified. Our present study in epithelial cells indicates that the synaptophysin-containing vesicles are not a homogeneous population. Both our vesicle fractionation and immunolocalization results show that (*a*) synaptophysin occurs in different kinds of vesicles in the same cell, often side-by-side, including occasional multivesicular bodies; (*b*) a large proportion of synaptophysin is contained in SET vesicles indistinguishable from typical SLMV; (*c*) that a subpopulation of synaptophysin-rich vesicles without detectable endocytotic markers can be distinguished from other vesicles that lack synaptophysin but contain markers of the endocytic recycling pathway; and (*d*) these cells also contain, in varying proportions, vesicles of considerably variable sizes in which synaptophysin colocalizes with endocytotic markers such as TFR (the latter observation being in agreement with observations in other cell types [2, 3, 6, 19–23, 26, 37]).

We do not at all exclude the possibility that the synaptophysin-rich, SLMV-type vesicles abundant in the transfected cells studied also contain a few TFR molecules, as suggested by Linstedt and Kelly (28) and also indicated in some of our immunolabeling experiments. We consider, however, the conclusion inescapable that the demonstrated synaptophysin enrichment over other molecules, TFR included, in one type of vesicle in the transfected epithelial cells described here must be the result of a positive sorting specific for synaptophysin, and that these synaptophysin-rich SLMV are novel structures in these cells since they greatly differ from vesicles of untransfected cells. Therefore, these transfected non-NE epithelial cells appear to represent an essentially similar situation as NE cells such as those of line PC12: a large proportion of the synaptophysin appears in SLMV with little, if any, endosomal markers, whereas another portion appears in vesicles of a broader size range that also contain these endosomal markers. Hence, sorting of the NE marker synaptophysin is not exclusive to NE cells, a conclusion at variance with those of other authors from other transfected nonepithelial cells (for references see above).

Perhaps non-NE cells can differ in their potential to sort effectively NE-typical proteins, and certain epithelial cells such as those studied by us are more competent in vesicle sorting of molecules usually characteristic of NE differentiation than other kinds of non-NE cells. Clearly, glycoproteins of the synaptophysin family are not restricted to NE cells, as shown by our recent discovery that many different kinds of non-NE cells, epithelial ones included, contain pantophysin, a protein closely related to synaptophysin, but slightly smaller than it (24). Consequently, the synaptophysin derived from the transfected gene in our experiments meets the similar endogenous pantophysin that may serve as a "sorting guide." In this connection, it should also be remembered that NE differentiation is within the pluripotential spectrum of many different epithelial "stem" cells and carcinomas, respectively, including examples of stratified tissues, as relevant for A-431 cells, and of simple epithelial cells, as relevant for PLC and SK-Hep-1 cells, including the differentiation of normal NE cells or of carcinoid tumors (for liver see, e.g., 27, 40, 41, 49, 57). Therefore, certain epithelium-derived cells may contain more or better components that help to sort NE membrane proteins, and only careful comparisons will help clarifying the importance of cell type "competence" for stabilizing and correctly accommodating a "foreign" NE protein introduced.

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References

1. Ajioka, R. S., and J. Kaplan. 1986. Intracellular pools of transferrin receptors result from constitutive internalization of unoccupied receptors. *Proc. Natl. Acad. Sci. USA.* 83:6445–6449.
2. Cameron, P. L., T. C. Südhof, R. Jahn, and P. De Camilli. 1991. Colocalization of synaptophysin with transferrin receptors: implications for synaptic vesicle biogenesis. *J. Cell Biol.* 115:151–164.
3. Clift-O'Grady, L., A. D. Linstedt, A. W. Lowe, E. Grote, and R. B. Kelly. 1990. Biogenesis of synaptic vesicle-like structures in a pheochromocytoma cell line PC-12. *J. Cell Biol.* 110:1693–1703.
4. Cutler, D. F., and L. P. Cramer. 1990. Sorting during transport to the surface of PC12 cells: divergence of synaptic vesicle and secretory granule proteins. *J. Cell Biol.* 110:721–730.
5. De Camilli, P., and R. Jahn. 1990. Pathways to regulated exocytosis in neurons. *Annu. Rev. Physiol.* 52:625–645.
6. Feany, M. B., A. G. Yee, M. L. Delvy, and K. M. Buckley. 1993. The synaptic vesicle proteins SV2, synaptotagmin, and synaptophysin are sorted to separate cellular compartments in CHO fibroblasts. *J. Cell Biol.* 123:575–584.
7. Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G.M. Ringold, and M. Danielsen. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl.*

Acad. Sci. USA. 84:7413–7417.
8. Franke, W. W., C. Grund, M. Osborn, and K. Weber. 1978. The intermediate-sized filaments in rat kangaroo PtK_2 cells. I. Morphology in situ. *Cytobiology.* 17:365–391.
9. Franke, W. W., C. Grund, and T. Achtstätter. 1986. Coexpression of cytokeratins and neurofilament proteins in a permanent cell line: cultured rat PC12 cells combine neuronal and epithelial features. *J. Cell Biol.* 103:1933–1943.
10. Grässel, S., A. Röling, and A. Hasilik. 1989. Immunoprecipitation of labeled antigens with Eupergit C1Z. *Anal. Biochem.* 180:72–78.
11. Green, S. A., and R. B. Kelly. 1992. Low density lipoprotein receptor and cation-independent mannose 6-phosphatase receptor are transported from the cell surface to the Golgi apparatus at equal rates in PC12 cells. *J. Cell Biol.* 117:47–55.
12. Hanover, J. A., and R. B. Dickson. 1985. Transferrin: receptor-mediated endocytosis and iron delivery. *In* Endocytosis. I. Pastan and M. C. Willingham, editors. Plenum Publishing Corp., New York. pp. 131–161.
13. Harding, C., J. Heuser, and P. Stahl. 1983. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J. Cell Biol.* 97:329–339.
14. Heuser, J. E., and S. Reese. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell Biol.* 57:315–344.
15. Hopkins, C. R. 1983. Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A431 cells. *Cell.* 35:321–330.
16. Hopkins, C. R., A. Gibson, M. Shipman, and K. Miller. 1990. Movement of internalized ligand-receptor complexes along a continuous endosomal reticulum. *Nature (Lond.).* 346:335–339.
17. Horst, M., N. Harth, and A. Haselik. 1991. Biosynthesis of glycosylated human lysozyme mutants. *J. Biol. Chem.* 266:13914–13919.
18. Hughson, E. J., and C. R. Hopkins. 1990. Endocytic pathways in polarized Caco cells: identification of an endosomal compartment accessible from both apical and basolateral surfaces. *J. Cell Biol.* 110:337–348.
19. Johnston, P. A., P. L. Cameron, H. Stukenbrok, R. Jahn, P. De Camilli, and T. C. Südhof. 1989. Synaptophysin is targeted to similar microvesicles in CHO and PC12 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 8: 2863–2872.
20. Johnston, P. A., and T. C. Südhof. 1990. The multisubunit structure of synaptophysin. Relationship between disulfide bonding and homo-oligomerization. *J. Biol. Chem.* 265:8869–8873.
21. Kelly, R. B. 1991. Secretory granule and synaptic vesicle formation. *Curr. Opin. Cell Biol.* 3:654–660.
22. Kelly, R. B. 1993. Storage and release of neurotransmitters. *Cell/Neuron.* 72/10(Suppl.):43–53.
23. Kelly, R. B., and E. Grote. 1993. Protein targeting in the neuron. *Annu. Rev. Neurosci.* 16:95–127.
24. Leube, R. E. 1994. Expression of the synaptophysin gene family is not restricted to neuronal and neuroendocrine differentiation in rat and human. *Differentiation.* 56:163–171.
25. Leube, R. E., P. Kaiser, A. Seiter, R. Zimbelmann, W. W. Franke, H. Rehm, P. Knaus, P. Prior, H. Betz, H. Reinke, K. Beyreuther, and B. Wiedenmann. 1987. Synaptophysin: molecular organization and mRNA expression as determined from cloned cDNA. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3261–3268.
26. Leube, R. E., B. Wiedenmann, and W. W. Franke. 1989. Topogenesis and sorting of synaptophysin: synthesis of a synaptic vesicle protein from a gene transfected into nonneuroendocrine cells. *Cell.* 59:433–446.
27. Lewin, K. 1987. Carcinoid tumors and the mixed (composite) glandular-endocrine cell carcinomas. *Am. J. Surg. Pathol.* 11(Suppl. 1):71–86.
28. Linstedt, A. D., and R. B. Kelly. 1991. Synaptophysin is sorted from endocytotic markers in neuroendocrine PC12 cells but not transfected fibroblasts. *Neuron.* 7:309–317.
29. Lowe, A. W., L. Madeddu, and R. B. Kelly. 1988. Endocrine secretory and neuronal synaptic vesicles have three integral membrane proteins in common. *J. Cell Biol.* 106:51–59.
30. MacNab, G. M., J. J. Alexander, G. Lecatsas, E. M. Bey, and J. M. Urbanowicz. 1976. Hepatitis B surface antigen produced by a human hepatoma cell line. *Br. J. Cancer.* 34:509–515.
31. Matteoli, M., K. Takei, M. S. Perin, T. C. Südhof, and P. De Camilli. 1992. Exo-endocytotic recycling of synaptic vesicles in developing processes of cultured hippocampal neurons. *J. Cell Biol.* 117:849–861.
32. Maycox, P. R., E. Link, A. Reetz, S. A. Morris, and R. Jahn. 1992. Clathrin-coated vesicles in nervous tissue are involved primarily in synaptic vesicle recycling. *J. Cell Biol.* 118:1379–1388.
33. Navone, F., R. Jahn, G. Di Gioia, H. Stukenbrok, P. Greengard, and P. De Camilli. 1986. Protein p38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. *J. Cell Biol.* 103: 2511–2527.
34. Orberger, G., R. Geyer, S. Stirm, and R. Tauber. 1992. Structure of the N-linked oligosaccharides of the human transferrin receptor. *Eur. J. Biochem.* 205:257–267.
35. Pang, D. T., J. K. T. Wang, F. Valtorta, F. Benfenati, and P. Greengard. 1988. Protein tyrosine phosphorylation in synaptic vesicles. *Proc. Natl. Acad. Sci. USA.* 85:762–766.
36. Pastan, I., and M. C. Willingham. 1985. The pathway of endocytosis. *In* Endocytosis. I. Pastan and M. C. Willingham, editors. Plenum Publishing Corp., New York. pp. 1–44.
37. Reetz, A., M. Solimena, M. Matteoli, F. Folli, K. Takei, and P. De Camilli. 1991. GABA and pancreatic β-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1275–1284.
38. Régnier-Vigouroux, A., S. A. Tooze, and W. B. Huttner. 1991. Newly synthesized synaptophysin is transported to synaptic-like microvesicles via constitutive secretory vesicles and the plasma membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3589–3601.
39. Rosewicz, S., D. Vogt, N. Harth, C. Grund, W. W. Franke, S. Ruppert, E. Schweitzer, E.-O. Riecken, and B. Wiedenmann. 1992. An amphicrine pancreatic cell line: AR42J cells combine exocrine and neuroendocrine properties. *Eur. J. Cell Biol.* 59:80–91.
40. Roskams, T., J. J. van den Oord, R. De Vos, and V. J. Desmet. 1990. Neuroendocrine features of reactive bile ductules in cholestatic liver disease. *Am. J. Pathol.* 137:1019–1025.
41. Roskams, T., R. De Vos, J. van den Oord, and V. J. Desmet. 1991. Cells with neuroendocrine features in regenerating human liver. *Acta Pathol. Microbiol. Immunol. Scand. Suppl.* 23:32–39.
42. Südhof, T. C., F. Lottspeich, P. Greengard, E. Mehl, and R. Jahn. 1987. A synaptic vesicle protein with a novel cytoplasmic domain and four transmembrane regions. *Science (Wash. DC).* 238:1142–1144.
43. Südhof, T. C., and R. Jahn. 1991. Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron.* 6:665–677.
44. Thomas, L., K. Hartung, D. Langosch, H. Rehm, E. Bamberg, W. W. Franke, and H. Betz. 1988. Identification of synaptophysin as a hexameric channel protein of the synaptic vesicle membrane. *Science (Wash. DC).* 242:1050–1053.
45. Thomas-Reetz, A. C., and P. De Camilli. 1994. A role for synaptic vesicles in non-neuronal cells: clues from pancreatic β cells and from chromaffin cells. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 8:209–216.
46. Tixier-Vidal, A., A. Faivre-Bauman, R. Picart, and B. Wiedenmann. 1988. Immunoelectron microscopic localization of synaptophysin in a Golgi subcompartment of developing hypothalamic neurons. *Neuroscience.* 26:847–861.
47. Tooze, J., and M. Hollinshead. 1992. In AtT20 and HeLa cells brefeldin A induces the fusion of tubular endosomes and changes their distribution and some of their endocytic properties. *J. Cell Biol.* 118:813–830.
48. Valtorta, F., R. Jahn, R. Fesce, P. Greengard, and B. Ceccarelli. 1988. Synaptophysin (p38) at the frog neuromuscular junction: its incorporation into the axolemma and recycling after intense quantal secretion. *J. Cell Biol.* 107:2717–2727.
49. Van der Wal, A. C., D. J. van Leeuwen, and N. Walford. 1990. Small cell neuroendocrine (oat cell) tumour of the common bile duct. *Histopathology.* 16:398–400.
50. Watts, C. 1985. Rapid endocytosis of the transferrin receptor in the absence of bound transferrin. *J. Cell Biol.* 100:633–637.
51. Weissman, A. M., R. D. Klausner, K. Rao, and J. B. Harford. 1986. Exposure of K562 cells to anti-receptor monoclonal antibody OKT9 results in rapid redistribution and enhanced degradation of the transferrin receptor. *J. Cell Biol.* 102:951–958.
52. Wiedenmann, B., and W. W. Franke. 1985. Identification and localization of synaptophysin, an integral membrane glycoprotein of M_r 38,000 characteristic of presynaptic vesicles. *Cell.* 41:1017–1028.
53. Wiedenmann, B., K. Lawley, C. Grund, and D. Branton. 1985. Solubilization of proteins from bovine brain coated vesicles by protein perturbants and Triton X-100. *J. Cell Biol.* 101:12–18.
54. Wiedenmann, B., W. W. Franke, C. Kuhn, R. Moll, and V. E. Gould. 1986. Synaptophysin: a marker protein for neuroendocrine cells and neoplasms. *Proc. Natl. Acad. Sci. USA.* 83:3500–3504.
55. Wiedenmann, B., H. Rehm, M. Knierim, and C.-M. Becker. 1988. Fractionation of synaptophysin-containing vesicles from rat brain and cultured PC12 pheochromocytoma cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 240:71–77.
56. Woods, J. W., M. Doriaux, and M. G. Farquhar. 1986. Transferrin receptors recycle to *cis* and middle as well as *trans*-Golgi cisternae in Ig-secreting myeloma cells. *J. Cell Biol.* 103:277–286.
57. Yu-Ping, X., and Y. Ji-yao. 1986. Primary neuroendocrine carcinoma of the liver. *Ultrastruct. Pathol.* 10:331–336.