Cellubrevin and Synaptobrevins: Similar Subcellular Localization and Biochemical Properties in PC12 Cells

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Abstract. There is strong evidence to indicate that proteins of the synaptobrevin family play a key role in exocytosis. Synaptobrevin 1 and 2 are expressed at high concentration in brain where they are localized on synaptic vesicles. Cellubrevin, a very similar protein, has a widespread tissue distribution and in fibroblasts is localized on endosome-derived, transferrin receptor-positive vesicles. Since brain cellubrevin is not detectable in synaptic vesicles, we investigated whether cellubrevin and the synaptobrevins are differentially targeted when co-expressed in the same cell. We report that in the nervous system cellubrevin is expressed at significant levels only by glia and vascular cells. However, cellubrevin is coexpressed with the two synaptobrevins in PC12 cells, a neuroendocrine cell line which contains synaptic vesicle-like microvesicles. In PC12 cells, cellubrevin has a distribution very similar to that of synaptobrevin 1 and 2. The three proteins are targeted to neurites which exclude the transferrin receptor and are enriched in synaptic-like microvesicles and dense-core granules. They are recovered in the synaptic-like microvesicle peak of glycerol velocity gradients, have a similar distribution in isopycnic fractionation and are coprecipitated by anti-synaptobrevin 2 immunobeads. Finally, cellubrevin, like the synaptobrevins, interact with the neuronal t-SNAREs syntaxin 1 and SNAP-25. These results suggest that cellubrevin and the synaptobrevins have similar function and do not play a specialized role in constitutive and regulated exocytosis, respectively.

A convergence of studies carried out in a variety of experimental systems has demonstrated that small proteins present at the cytoplasmic surfaces of transport vesicles and of target membranes play a key role in membrane docking and fusion (Söllner et al., 1993*a*,*b*; Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994). These proteins, called v- and t-SNAREs respectively, because they act as SNAP receptors, have cytoplasmic domains with short membrane anchors (Söllner et al., 1993*a*,*b*). Synaptobrevin 1 and 2 (VAMP1 and 2) are v-SNAREs expressed at high concentrations in neurons where they are concentrated on synaptic vesicles (Trimble et al., 1988, 1990; Baumert et al., 1989). Their selective cleavage by the light chains of tetanus toxin and of botulinum toxins B, D, and F is responsible for the potent block of neurotransmitter release produced by these toxins (Schiavo et al., 1992, 1993; Yamasaki et al., 1994).

A protein which shares a high degree of similarity with synaptobrevin 1 and 2, referred to as cellubrevin, is expressed in a wide variety of tissues (McMahon et al., 1993). In fibroblasts, this protein was found to be closely colocalized with vesicles which recycle transferrin (Tf)[1] receptor (McMahon et al., 1993; Galli et al., 1994). Cellubrevin, like the two synaptobrevins, is a substrate for the proteolytic action of tetanus toxin (McMahon et al., 1993; Galli et al., 1994; Yamasaki et al., 1994), and its cleavage in SLO-permeabilized cells impairs the constitutive exocytosis of endosome-derived vesicles (Galli et al., 1994).

The identification of families of v- and t-SNARES (Elferink et al., 1989; Bennett and Scheller, 1993; Bennett et al., 1993; Chin et al., 1993; McMahon et al., 1993; DiAntonio et al., 1993) has raised the possibility that the multiplicity of these proteins may be responsible for conferring specificity to exocytosis of distinct vesicular carriers. In any given cell, the presence of distinct v-SNAREs on different types of secretory vesicles may play a role in defining tem-

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1. *Abbreviations used in this paper*: TeTx, tetanus toxin; TeTx-LC, tetanus toxin light chain; Tf, transferrin; SL-O, streptolysin-O.

poral and spatial parameters of exocytosis. We have previously shown that in neurons the Tf receptor is excluded from axons and synaptic vesicles, and is restricted to somatodendritic regions (Cameron et al., 1991; Mundigl et al., 1993). It was therefore of interest to determine whether cellubrevin is expressed in cells which contain synaptic vesicles and whether it is targeted to these organelles or is restricted to Tf receptor containing vesicles.

Although cellubrevin is expressed in the nervous system (McMahon et al., 1993), we report here that in brain it is localized primarily in supportive cells (glial, endothelial, and ependymal cells). However, cellubrevin is expressed together with synaptobrevin 1 and 2 in neuroendocrine PC12 cells, which contain synaptic-like microvesicles. In these cells, cellubrevin is colocalized with synaptobrevin 1 and 2 and interacts with the same neuronal t-SNAREs, syntaxin 1, and SNAP-25. These findings speak against a specialized role of cellubrevin for constitutive secretion and suggest that the three v-SNAREs have similar function.

Materials and Methods

Materials

[^{125}I]Protein A (8.8 μCi/μg) was purchased from NEN (Wilmington, DE), [^{3}H]norepinephrine (238 μCi/μg) and ECL detection reagents were from Amersham Corp. (Arlington Heights, IL), reduced streptolysin-O (SL-O) from Murex Diagnostics (Norcross, GA), ATP (magnesium salt) from Sigma Chem. Co. (St. Louis, MO), and human diferric Tf from GIBCO BRL (Gaithersburg, MD). Tissue culture reagents were from GIBCO BRL. The recombinant light chain of tetanus toxin (TeTx-LC) was a kind gift from Dr. H. Niemann (Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Germany). A stock solution of TeTx-LC was prepared as described by McMahon et al. (1993) at a concentration of 13 μM in buffer T (10 mM Pipes, 150 mM K-glutamate, pH 7.2).

Antibodies

Antibodies specific for synaptophysin (MC1), synaptobrevin 2 (MC 23), and SNAP-25 (MC21) were generated in rabbits by injecting synthetic peptide corresponding to the following amino acid sequences of the three proteins flanked by an additional cysteine (shown in italics): amino acids 258-274 of synaptophysin, GGYQPDYGQPASGGGGYC, amino acids 1-16 of synaptobrevin 2, MSATAATVPPAAPAGEC, amino acids 195-206 of SNAP-25A, CANQRATKMLGSG. The additional cysteine was used for conjugation to keyhole limpet hemocyanin (Calbiochem-Behring Corp., La Jolla, CA). The sera were affinity purified using the peptide conjugated to EAH Sepharose (Pharmacia, Uppsala, Sweden). Rabbit antibodies specific for synaptobrevin 1 (MC9), cellubrevin (MC16), and synaptotagmin 1 (8907) were described in earlier studies (Galli et al., 1994; Mundigl et al., 1993). The specificity of the antibodies for synaptobrevin 1 and 2 and cellubrevin was confirmed by Western blotting of these proteins expressed as recombinant fusion proteins and in rat brain and PC12 cell extracts. In all cases the three antibodies recognized selectively bands with the expected differences in electrophoretic mobility in Schagger gels (see below). The following antibodies were generous gifts: monoclonal antibodies against synaptobrevin 2 (Cl 69.1) (Edelmann et al., 1994) and synaptotagmin 1 (Cl41.1) (Matteoli et al., 1992) from Dr. R. Jahn (Yale University, New Haven, CT), monoclonal antibody against syntaxin 1 (HPC-1) from Dr. C. Barnstable (Yale University), monoclonal antibody against the 30-kD ADP/ATP translocator of the inner mitochondrial membrane from Dr. W. Neupert (University of Munich, Munich, Germany), rabbit sera directed against secretogranin 1 and 2 (Rosa et al., 1985) from Dr. P. Rosa (Milano, Italy) and monoclonal antibodies anti-human Tf receptor (No. H68.4) from Dr. I. Trowbridge (Salk Institute, La Jolla, CA). Indocarbocyanine (Cy3)- and fluorescein (DTAF)-conjugated goat anti-rabbit and -mouse IgGs were from Jackson Immuno-Research Labs (West Grove, PA). Nonimmune rabbit IgGs and rabbit anti-mouse IgGs were from Cappel (Cochranville, PA), anti-Tf monoclonal antibodies were from Accurate Chemical and Scientific Corp. (Westbury, NY), anti-synaptophysin monoclonal antibodies (SVP38) from Sigma Chem. Co.

Hippocampal Cell Cultures

Neurons were grown on glass coverslips as described by Banker and Cowan (1977) and Bartlett and Banker (1984). Briefly, hippocampi from 18-d-old fetal rats were dissociated by treatment with trypsin (0.25% for 15 min at 37°C), followed by trituration with a fire-polished Pasteur pipette. Dissociated cells were plated on poly-L-lysine-treated glass coverslips in MEM with 10% horse serum at densities ranging from 1,000–4,000 cells/cm^2. After 3 h, cortical glial cells (Booher and Sensenbrenner, 1972) were plated at a density of 1,500 cells/cm^2 on top of the neurons. After attachment of the glial cells, coverslips were then transferred to dishes containing a monolayer of cortical glial cells (Booher and Sensenbrenner, 1972), so that they were suspended over the glial cells but not in contact with them (Booher and Sensenbrenner, 1972). Cells were maintained in MEM without sera, supplemented with 1% HL1 (Ventrex, Portland, ME), 2 mM glutamine and 1 mg/ml BSA (neuronal medium) and used for immunofluorescence after 4–5 d in vitro.

PC12 Cell Cultures

PC12 cells (Greene and Tischler, 1976) were grown as monolayer cultures on collagen-coated tissue culture dishes in DME supplemented with 10% fetal bovine serum, 5% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C in 7.5% CO_2. For NGF differentiation, PC12 cells were first primed for 2 wk with 50 ng/ml NGF 2.5S (from rat submaxillary gland, grade II; Boehringer-Mannheim Biochemicals, Indianapolis, IN) in DME supplemented with 1% horse serum in 7.5% CO_2. Cells were then squirted from the dish with fresh media and pelleted by centrifugation for 5 min at 250 *g*. To reduce the number of cell aggregates, pellets were resuspended in 0.25% trypsin-EDTA (Sigma Chem. Co.) and incubated for 15 min at 37°C. They were then pelleted and resuspended in serum-containing medium (see above), dissociated with a fire polished Pasteur pipet and plated on laminin-coated coverslips at a density of 7,500–15,000 cells/cm^2. After 2–4 h of incubation at 37°C, the coverslips were inverted and placed over a bed of glial cells as previously described for hippocampal neurons (Goslin and Banker, 1990). The cells were maintained in neuronal medium, in 5% CO_2, with no NGF added, and used after 3–5 d in vitro.

Immunofluorescence

Rat brain fixation, preparation of frozen sections and immunofluorescence was performed as previously described (De Camilli et al., 1983; Takei et al., 1992). PC12 cells were fixed with 4% formaldehyde in phosphate buffer containing 4% sucrose and processed for double immunofluorescence as previously described (Cameron et al., 1991; Mundigl et al., 1993). In some cases, prior to fixation cells were incubated for 1 h at 37°C in buffer K (20 mM Hepes, 128 mM NaCl, 3 mM KCl, 1 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 2.7 mM $CaCl_2$, and 11 mM glucose, pH 7.4, with NaOH) containing 50 μg/ml human diferric Tf. They were then briefly washed with cold buffer and fixed.

Glycerol and Sucrose Gradients

Velocity centrifugation on glycerol density gradients of PC12 cells and RINm5 cells homogenates (Gazdar et al., 1980) was performed as described by Cameron et al. (1991). The synaptobrevin and cellubrevin content of the various fractions was analyzed by immunoprecipitation followed by Western blotting. Sucrose equilibrium density gradient centrifugation of post nuclear supernatants of PC12 cells and bovine chromaffin cells was performed as described by Navone et al. (1989).

Immunobead Isolation of Organelles

Monoclonal antibodies Cl 69.1 or SVP38 were covalently coupled to Eupergit C1Z immunobeads (1-μm mean diam; Roehm Pharma, Darmstadt, Germany) as described (Burger et al., 1989). PC12 cells were washed in buffer K, pelleted and resuspended at a density of 15 × 10^6 cells/ml in homogenization buffer (0.25 M sucrose, 4.0 mM Hepes, pH 7.2, 1 mM $MgCl_2$, 0.4 mM PMSF, 1 mM benzamidine, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The cells were then passed 20 times through a ball bearing cell cracker (Bernie-tech Engineering, Saratoga, CA) with a 0.0006 in. clearance and the lysate was centrifuged for 20 min at 10,000 *g*. Supernatants (containing ~2 mg/ml protein) were incubated with anti-synaptophysin or anti-synaptobrevin 2 beads, respectively, for 4 h at 4°C under rotation. A ratio of immunobeads to cell extract was used sufficient to deplete all synaptobrevin 2-positive organelles from

the supernatant. The beads were sedimented for 6 min at 1,300 *g* and washed three times with homogenization buffer.

Electron Microscopy of Immunobead Adsorbed Organelles

A PC12 cell lysate was obtained as described above and centrifuged at 676 *g* for 15 min. Supernatants were incubated with anti-synaptobrevin 2 or control mouse IgGs immunobeads for 3 h at 4°C under rotation to allow adsorption of synaptobrevin 2 containing organelles to the immunobeads. The samples were fixed with 3% paraformaldehyde, 2% glutaraldehyde, 0.25 M sucrose in 5 mM Hepes-KOH, pH 7.4, for 1 h, centrifuged for 3 min at 13,800 *g*. The beads were resuspended in 0.12 M Na-phosphate buffer and embedded in 1% agarose. The agarose blocks were postfixed with 1% OsO_4 in 0.1 M Cacodylate, pH 7.4, dehydrated, embedded in EPON812, and cut in thin sections (30 nm). The samples were analyzed on a Philips CM10 microscope.

Immunoprecipitation of the SNARE Complexes

PC12 cells were washed once with buffer K. They were then resuspended in solubilization buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 10 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml aprotinin) at a concentration of 1–2 mg/ml. Triton X-100 was then added at a final concentration of 1% and the resulting cell extract was incubated for one hour at 4–6°C under rotation and subsequently centrifuged at 200,000 *g* for 15 min. The supernatant was incubated overnight at 4–6°C under rotation, with protein G sepharose beads (for mouse antibodies) or protein A (for rabbit antibodies) (Pharmacia, Piscataway, NJ) precoated with the following antibodies: monoclonal anti-synaptobrevin 2 (Cl 69.1), monoclonal anti-syntaxin 1 (HPC-1), mouse control IgGs, rabbit serum directed against SNAP-25 (MC21), control rabbit IgGs. The beads were then pelleted for 1 min at 14,000 *g* and washed three times with solubilization buffer containing 1% Triton X-100. Finally, the beads were resuspended in gel sample buffer and boiled for 5 min.

Norepinephrine Release from Streptolysin-O–perforated Cells

Streptolysin-O (SL-O) was used to perforate cells as previously described (Ahnert-Hilger et al., 1989; Miller and Moore, 1992). Approximately 40×10^6 PC12 cells were resuspended in RPMI media supplemented with 1 mM ascorbic acid at a density of 2×10^6 cells/ml and were incubated with 2 μCi/ml of [^{3}H]norepinephrine (NE) for 2 h at 37°C and 7.5% CO_2. At the end of the incubation, cells were washed with 1 ml of ice-cold buffer K (see above) by sedimentation and resuspension using an Eppendorf 5154C microcentrifuge. The cells were then washed two times with 1 ml of ice-cold buffer A (20 mM Hepes, 110 mM NaCl, 5.4 mM KCl, 0.9 mM Na_2HPO_4, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 11 mM glucose, pH 7.4, with NaOH), resuspended in buffer A to give a final concentration of 10^4 cells/μl and mixed with an equal volume of buffer A containing SL-O (0.3 IU/ml final concentration). After incubation in an ice-water bath for 10 min, excess SL-O was removed by centrifugation and one wash with 1 ml of buffer B (20 mM Hepes, 100 mM K-glutamate, 40 mM KCl, 5 mM EGTA, pH 7.2, with KOH). The cell suspension was split in two and both samples were pelleted. One sample was resuspended with 2 ml of buffer B containing 2 mM Mg-ATP and 1 mM free Mg^{2+} (added as $MgCl_2$) with no Ca^{2+} added, the other sample with 2 ml of the same medium but with 100 μM free Ca^{2+}(added as $CaCl_2$). The concentration of free Mg^{2+} and Ca^{2+} were calculated using the software by Föhr and Warchol (Ulm, FRG) (Föhr et al., 1993). Submicromolar concentrations of free Ca^{2+} were used to induce release because at these low concentrations Ca^{2+} triggers release only from permeabilized cells (Wilson and Kirshner, 1983). Each of the two samples was further split in two, and the light chain of tetanus toxin (TeTx-LC) was added to one of the two halves at the final concentration of 300 nM. 100-μl aliquots of each of the four cell suspensions were then incubated at 37°C for 0, 5, 15, and 25 min after which supernatants and pellets were collected separately. As previously reported, the SL-O–induced formation of cell pores occurs as soon as the cells are shifted to 37°C (Ahnert-Hilger et al., 1989). Triplicate samples were prepared for each time point. Pellets were immediately resuspended in 100 μl of SDS sample buffer and subsequently processed for SDS-PAGE and Western blotting. The amounts of [^{3}H]NE in the supernatants and corresponding pellets were measured in a β-counter (LKB, Bromma, Sweden). The amount of [^{3}H]NE recovered in the supernatant at the 0 time point was defined as background and therefore subtracted from each individual release value obtained at later incubation times. [^{3}H]NE secretion (supernatant) was expressed as the percent of the total amount of [^{3}H]NE present in each sample (supernatant plus pellet). Each experiment was repeated at least three times, and a single representative experiment is shown in each figure. Values are reported as the mean ± SD of three determinations.

In pilot experiments, the effectiveness of the permeabilization procedure for cells in suspension was assessed by incubating SL-O treated cells with propidium iodide (1 μg/ml) or fluorescein labeled phalloidin (50 nM) followed by flow cytometry analysis using a FACSTARplus cell sorter (Becton Dickinson, San Jose, CA) (excitation at 488 nm and emission at 630 and 530 nm, respectively). Over 90% of the cells were found to be perforated.

Western Blot Analysis

Homogenates of tissues and cells were prepared in 10 mM Hepes, pH 7.4, 150 mM NaCl, 0.1 mM $MgCl_2$, 1 mM EGTA plus protease inhibitors (1 μg/ml each of leupeptin, aprotinin, pepstatin, and antipain and 0.4 mM PMSF). Protein concentrations were determined using the procedure of Petersen (1977). SDS-polyacrylamide gel electrophoresis was performed according to Schagger and von Jagow (1987), a procedure which maximizes separation of low molecular weight proteins. Immunoblotting was performed as described by Towbin et al. (1979) using either ECL or [^{125}I] protein A as the detection system.

Results

Fig. 1 shows that synaptobrevin 1 and 2, as well as cellubrevin, as detected by rabbit anti-peptide antibodies specific for each of the three proteins, are expressed in brain. However, while synaptobrevin 1 and 2 were enriched in a synaptic vesicle preparation, cellubrevin, which is present at low concentrations in brain, was not detectable in the same synaptic vesicle fraction. To demonstrate whether this result is explained by differential targeting of cellubrevin and synaptobrevin 1 and 2 in neurons, we investigated the localization of the three proteins by immunofluorescence on frozen brain sections and in primary cultures of brain cells. These experiments demonstrated that cellubrevin immunoreactivity was concentrated in glial cells and other supportive cells of the nervous system, but was not detectable in neurons. Examples of the differential distribution of the synaptobrevins and cellubrevin are shown in Figs. 2 and 3. Synaptobrevin 1 and 2 immunoreactivities are highly concentrated in nerve terminals of the molecular layer and the granule cell layer, where they are colocalized with synaptophysin (Fig. 2, *a–d*). Cellubrevin, in contrast, is concentrated in non-neuronal cells including endothelial and ependymal cells (see Figs. 2, *e* and *f* and 3, *a–d*). In primary culture of brain cells, synaptobrevin 2 is highly concentrated in neurons while cellubrevin immunoreactivity is restricted primarily to glial cells (Fig. 3, *e* and *f*). In glial cells, cellubrevin was found to be closely colocalized with internalized Tf in the region of the Golgi complex and in vesicles throughout the cytoplasm (not shown) as described previously for CHO cells (Galli et al., 1994).

To determine if cellubrevin, like synaptobrevin 1 and 2, contains a signal which can target it to synaptic vesicles, it was important to identify a cell type which: (*a*) expresses all three proteins; and (*b*) contains synaptic vesicle-like organelles. PC12 cells were found to have these properties. The presence of organelles which are structurally and biochemically related to synaptic vesicles of neurons referred to as synaptic-like microvesicles, and which exclude Tf receptors, is well documented in these cells (Navone et al., 1986; Wiedenmann et al., 1988; Cameron et al., 1991; Linstedt and Kelly, 1991). As shown in Fig. 4, the synaptobrevins and

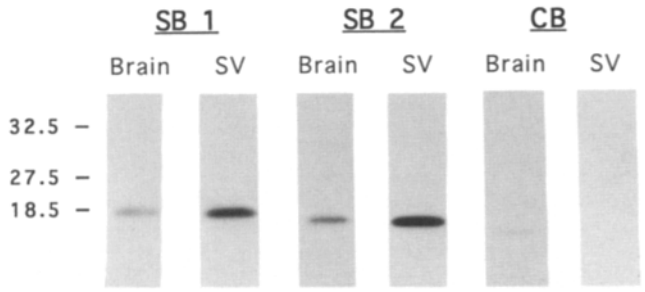

Figure 1. Enrichment of synaptobrevin 1 and 2, but not of cellubrevin, in synaptic vesicles of rat brain. Proteins in brain homogenate (Brain, 100 μg/lane) and purified synaptic vesicles (*SV*, 50 μg/lane) were separated by SDS-PAGE and probed by Western blotting for the presence of synaptobrevin 1 (*SB1*), synaptobrevin 2 (*SB2*), and cellubrevin (*CB*).

cellubrevin are expressed in PC12 cells. Synaptobrevin 1 and 2 are present at a much lower concentration in PC12 cells than in brain, while cellubrevin is present at a much higher concentration in PC12 cells (Fig. 4).

The distribution of the three proteins was investigated by immunofluorescence in PC12 cells which had been primed with NGF and then cultured, with no NGF added, on coverslips inverted over a monolayer of cortical astrocytes as described for hippocampal neurons (Goslin and Banker, 1990). Coculture with glial cells was found to be particularly effective in promoting neurite outgrowth (Fig. 5). In PC12 cells, not only synaptobrevin 1 and 2 immunoreactivities, but also cellubrevin immunoreactivity, were concentrated in neurites, which are enriched in synaptic-like microvesicles and dense-core secretory granules (Fig. 5). In addition, punctate immunoreactivity for all three proteins was present in the perikaryal region where a particularly high concentration of the proteins was localized in the area occupied by the Golgi complex. As shown by double immunofluorescence, the distribution of each of the three proteins was found to be very similar to the distribution of synaptophysin, a synaptic vesicle protein which in PC12 cells is concentrated in the membranes of synaptic-like microvesicles and endosomes (Cameron et al., 1991) (Fig. 5).

To determine whether cellubrevin immunoreactivity in neurites was due to the concentration of Tf-containing vesicles in these processes, rather than to the localization of cellubrevin in synaptic-like microvesicles and possibly dense-core secretory granules, PC12 cells were double labeled for synaptobrevin and Tf following a 1-h incubation with iron-saturated Tf. This procedure allows steady-state labeling of all Tf receptor-containing compartments (Cameron et al., 1991). As shown in Fig. 6, Tf immunoreactivity was restricted to cell bodies and was virtually absent from neurites

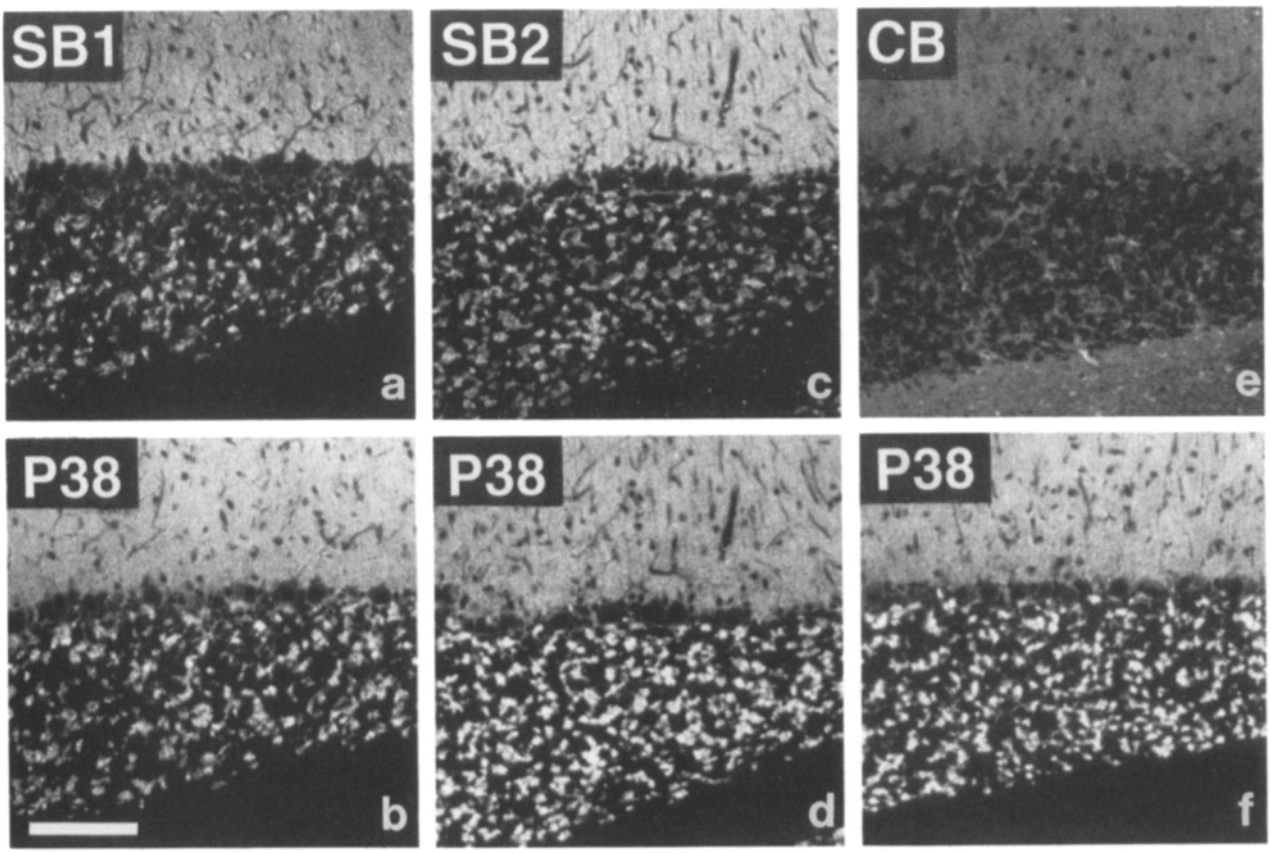

Figure 2. Differential localization of the two synaptobrevins and of cellubrevin in rat cerebellum. Frozen sections were processed by double immunofluorescence for each of the three proteins (*top*) and for synaptophysin (*bottom*). The distribution of synaptobrevin 1 (*SB1*) and synaptobrevin 2 (*SB2*) immunoreactivity is very similar to the distribution of synaptophysin immunoreactivity (*P38*). Cellubrevin immunoreactivity (*CB*) is primarily localized in endothelial cells and in glial cells of both gray and white matter. Bar, 100 μm.

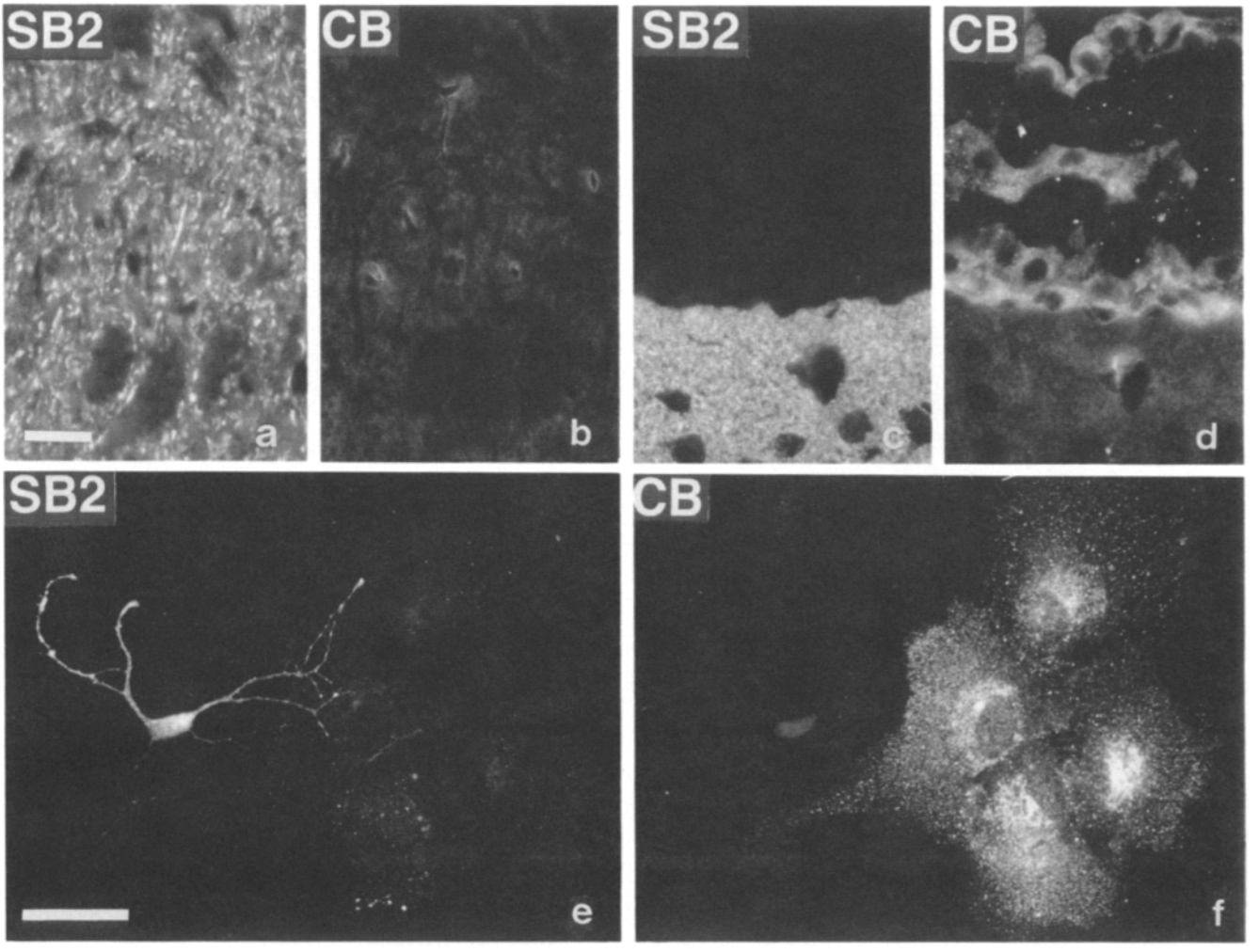

Figure 3. Differential localization of synaptobrevin 2 and cellubrevin in frozen sections of rat brain and primary culture of rat brain cells. (*Top*) Double immunofluorescence of brain stem (*a* and *b*) and cerebral cortex (*c* and *d*) for synaptobrevin 2 (*SB2*) and for cellubrevin (*CB*). Synaptobrevin 2 immunoreactivity has the typical nerve terminal pattern while cellubrevin is primarily localized in supportive cells (*b*) and cells of the choroid plexus (*d*). (*Bottom*) Double immunofluorescence of a coculture of neurons and astrocytes for synaptobrevin 2 (*SB2*) and cellubrevin (*CB*). Synaptobrevin 2 immunoreactivity is restricted to the neuron and cellubrevin to the astrocytes. Bars: (*A*) 20 μm; (*B*) 25 μm.

where both the synaptobrevins and cellubrevin were localized. However, it was not possible to discriminate from the fluorescent images whether cellubrevin was colocalized with synaptobrevin on the same organelles or whether it was localized on a separate population of endosomal membranes.

A unique property of synaptic-like microvesicles is their migration in glycerol velocity gradients as a peak distinct from that of endosomal membranes (Cameron et al., 1991; Linstedt and Kelly, 1991). Therefore, a low speed supernatant from an undifferentiated PC12 cell homogenate was analyzed by this method. Gradient fractions were probed by Western blotting for their cellubrevin, synaptobrevin 2, synaptophysin and Tf receptor content (Fig. 7). Early endosomes, as indicated by the distribution of the Tf receptor, were present only in the fractions at the bottom of the gradient which also contains a variety of other organelles including plasma membrane and dense-core secretory granules (Fig. 7, fractions *10–12*) (Cameron et al., 1991). As described previously, synaptophysin migrated as two well-defined peaks: an endosomal peak and a slower moving peak, corresponding to synaptic-like microvesicles (Fig. 7, peak in fraction *5*) (Cameron et al., 1991). A migration in two peaks, similar to that of synaptophysin but different from that of the Tf receptor, was observed for both synaptobrevin

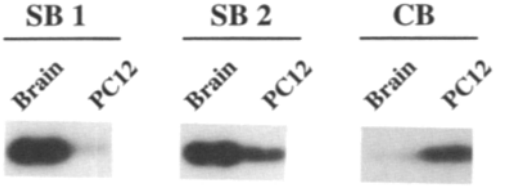

Figure 4. Comparison of the expression of synaptobrevin 1 and 2 and of cellubrevin in brain and PC12 cells. Proteins in homogenates of rat brain (25 μg/lane) and PC12 cells (50 μg/lane) were separated by SDS-PAGE and probed by Western blotting for the presence of synaptobrevin 1 (*SB1*), synaptobrevin 2 (*SB2*) and cellubrevin (*CB*).

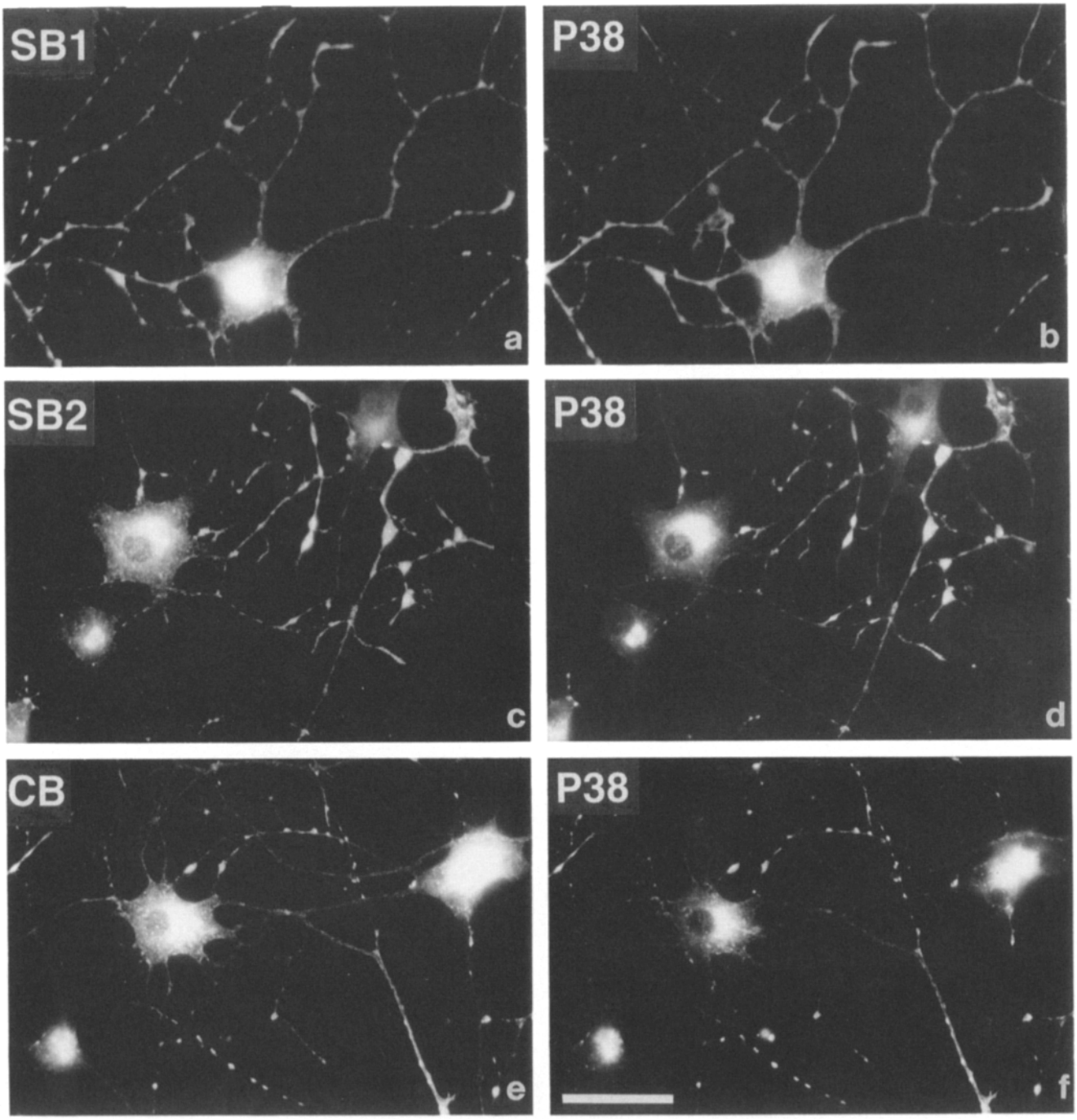

Figure 5. Concentration and colocalization of synaptobrevin 1 and 2 and cellubrevin with synaptophysin in neurites of NGF-differentiated PC12 cells. Double immunofluorescence for each of the three proteins (*SB1*, *SB2*, and *CB*) (*left*) and for synaptophysin (*P38*) (*right*). Bar, 50 μm.

2 and cellubrevin (Fig. 7). A similar sedimentation pattern in glycerol velocity gradients was observed for cellubrevin from low-speed postnuclear supernatants of RINm5 cells, a pancreatic endocrine cell line (data not shown). These results demonstrate the presence of cellubrevin in synaptic-like microvesicles in both PC12 and RINm5 cells.

The membrane distribution of synaptobrevin 1 and 2 and cellubrevin, was also compared in isopycnic centrifugation on sucrose density gradients (Fig. 8). This procedure allows separation of dense-core secretory granules from the peak fractions of synaptic-like microvesicles and other light membranes (such as endosomes and plasmalemma) (Cameron et al., 1991; Reetz et al., 1991). The migration in the gradient of synaptic-like microvesicles and endosomes was indicated by the presence of synaptophysin, and the migration of dense core granules by the presence of secretogranin 1, a secretory peptide (Rosa et al., 1985) (Fig. 8). The dense fractions of the gradient containing the granules were also highly enriched in synaptotagmin (Fig. 8) as previously observed (Walch-Solimena et al., 1993). The migrations of

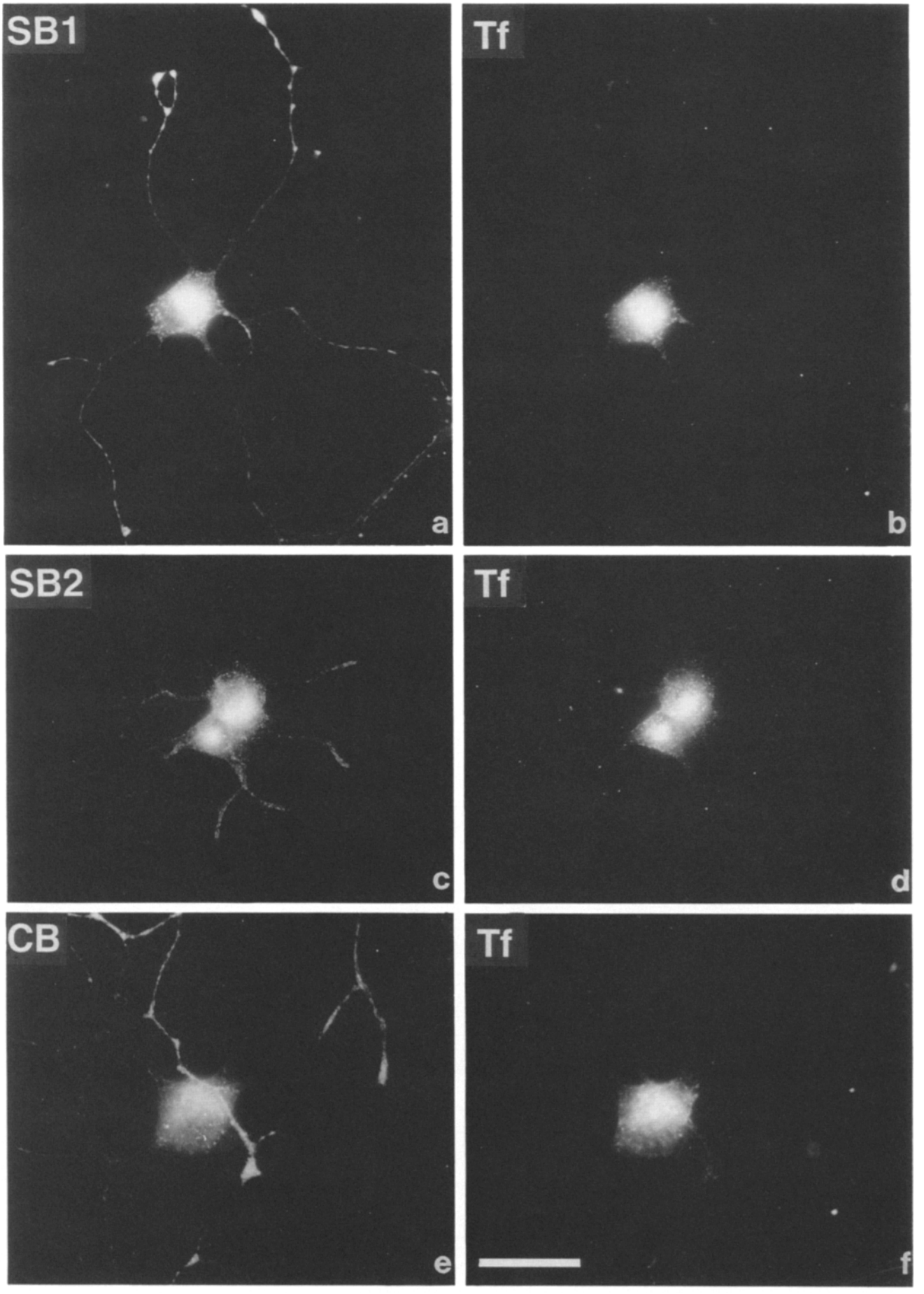

Figure 6. Concentration of synaptobrevins and cellubrevin, but not pre-internalized human Tf, in the neurites of NGF-differentiated PC12 cells. PC12 cells were incubated with human Tf (50 μg/ml) prior to fixation. They were then stained by double immunofluorescence for internalized Tf (*Tf*) and for synaptobrevin 1 (*SB1*), synaptobrevin 2 (*SB2*) and cellubrevin (*CB*). Bar, 50 μm.

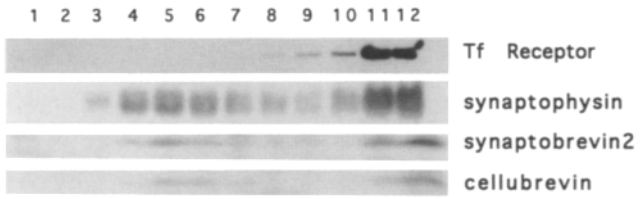

Figure 7. Distribution of endosomal and synaptic vesicle proteins from a low speed supernatant of PC12 cell homogenate on a glycerol velocity gradient. The low-speed supernatant was centrifuged on a 5–25% glycerol gradient with a 50% sucrose cushion at the bottom of the gradient. Equal volumes of each fraction (*Tf Receptor, synaptophysin*), or immunoprecipitates of equal volumes (*synaptobrevin 2, cellubrevin*), were analyzed by SDS-PAGE and Western blotting. The fractions are numbered starting from the top of the gradient. Synaptobrevin 2 and cellubrevin, but not Tf receptor, are present in the synaptic-like microvesicle peak (fractions *4–6*), identified by the presence of synaptophysin.

synaptobrevin 1 and 2 and of cellubrevin in the gradient were very similar, with the bulk of the three proteins being recovered in light fractions. Their migration was in turn similar to that of synaptophysin. However, a light trail of all these proteins overlapped with the dense-core granule containing fractions (Fig. 8).

Finally, to assess whether the three v-SNAREs are colocalized on the same vesicles, we carried out organelle immunoisolation experiments. A PC12 cell extract was incubated with anti-synaptobrevin 2 immunobeads and the presence of synaptobrevin 1 and cellubrevin on the immunoisolated organelles was assessed by Western blotting. Immunoisolation experiments with anti-synaptophysin immunobeads and non-immune mouse IgGs were carried out as controls. As shown in Fig. 9, the bulk of synaptobrevin 2 was recovered in the pellet fraction (P) using both anti-synaptophysin and anti-synaptobrevin 2 immunobeads. A similar enrichment in the pellets was observed for synaptobrevin 1 and cellubrevin and for other proteins of synaptic-like microvesicles, secretory granules and endosomes (synaptophysin, synaptotagmin 1, secretogranin 2 and transferrin receptor). The property of anti-synaptobrevin 2 immunobeads to deplete almost completely synaptobrevin 1 and cellubrevin from the PC12 cell extract demonstrates that nearly all vesicles positive for synaptobrevin 2 are also positive for synaptobrevin 1 and cellubrevin. Thus, the three proteins do not colocalize only on sorting compartments such as endosomes, but in most organelles. This result conclusively establishes that synaptobrevin 1 and 2 and cellubrevin are colocalized on the same vesicles. The mitochondrial ATPase remained in the supernatants confirming the specificity of the immunoisolation procedure (Fig. 9). All these proteins were recovered in the supernatants of the non-immune IgG beads. The complete immunoisolation of secretogranin 2 contrasts with the low amount of synaptobrevin and cellubrevin observed in the dense-core granule containing fractions of sucrose density gradients (Fig. 9). Electron microscopy analysis showed that in addition to a large number of pleomorphic vesicles, dense-core granules were also present on the synaptobrevin 2 immunobeads (Fig. 10). While the bulk of synaptophysin and synaptobrevin/cellubrevin is clearly localized on light vesicles, a small amount of these proteins present in the membranes of the granules may be sufficient to allow their immunoisolation.

It has been reported that TeTx-LC inhibits catecholamine release from dense-core vesicles in SLO-permeabilized PC12 cells (Ahnert-Hilger et al., 1989; Ahnert-Hilger and Weller, 1993). Our results indicating the presence of the

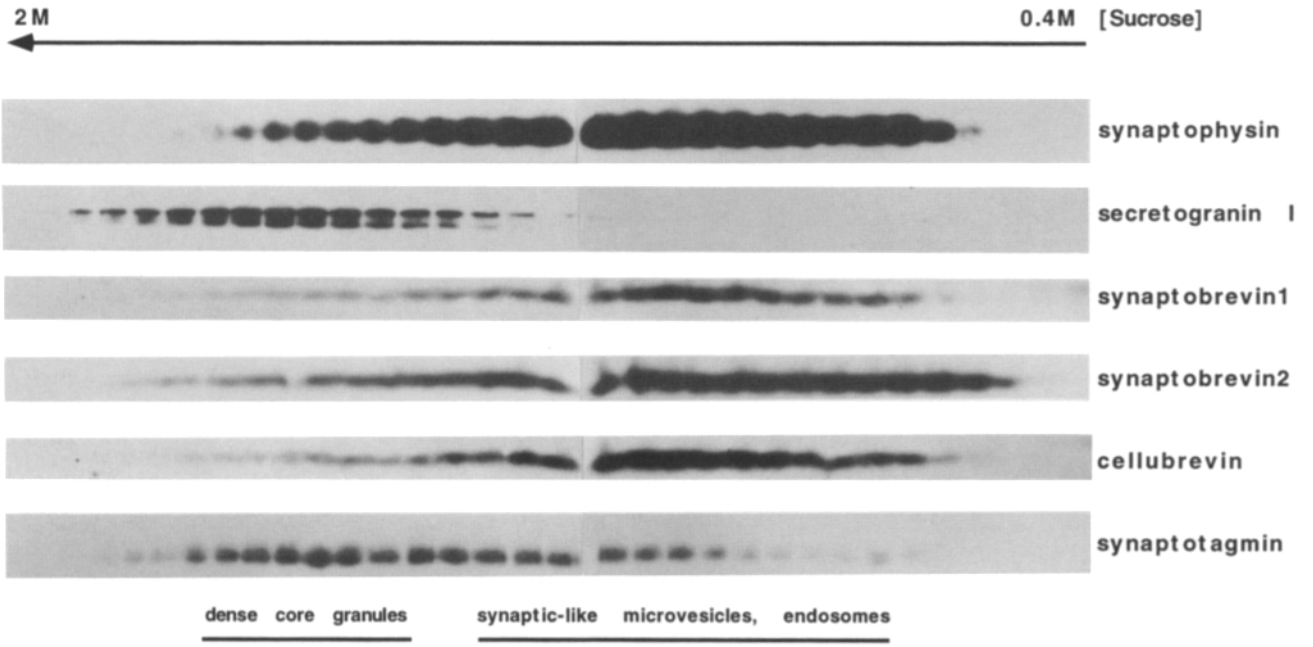

Figure 8. Distribution of secretory vesicle markers from a low-speed supernatant of PC12 cell homogenate by isopycnic centrifugation on a 0.4–2 M sucrose gradient. Equal volumes of gradient fractions were analyzed by SDS-PAGE and Western blotting for the proteins indicated.

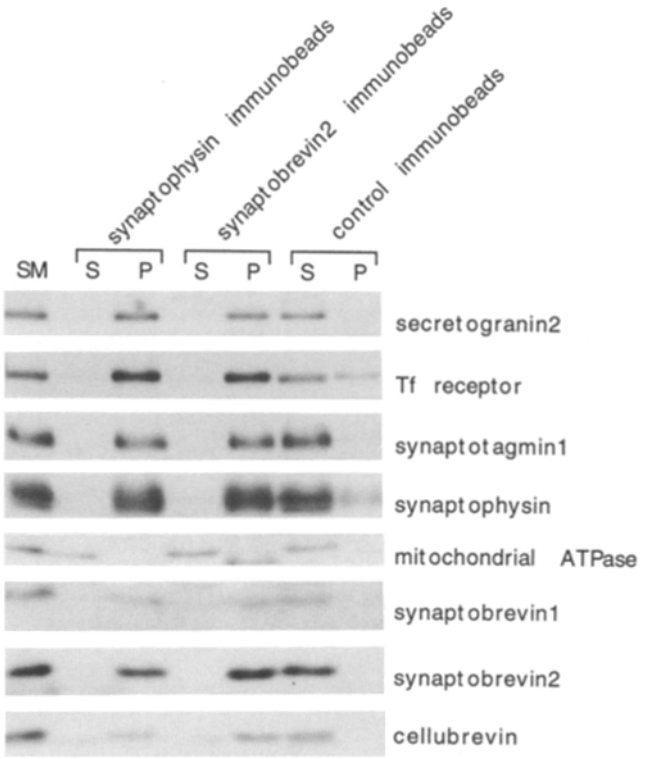

Figure 9. Immunoisolation of synaptophysin- or synaptobrevin 2-containing organelles. A high-speed postnuclear supernatant from PC12 cells was incubated with anti-synaptophysin, anti-synaptobrevin 2, or control mouse IgG metacrylate immunobeads. Equal volume of the starting material (*SM*), the supernatants (*S*), or the bead pellets (*P*) were analyzed by SDS-PAGE and Western blotting for their content in secretogranin 2, Tf receptor, synaptotagmin 1, synaptophysin, mitochondrial ATPase, synaptobrevin 1, synaptobrevin 2, and cellubrevin.

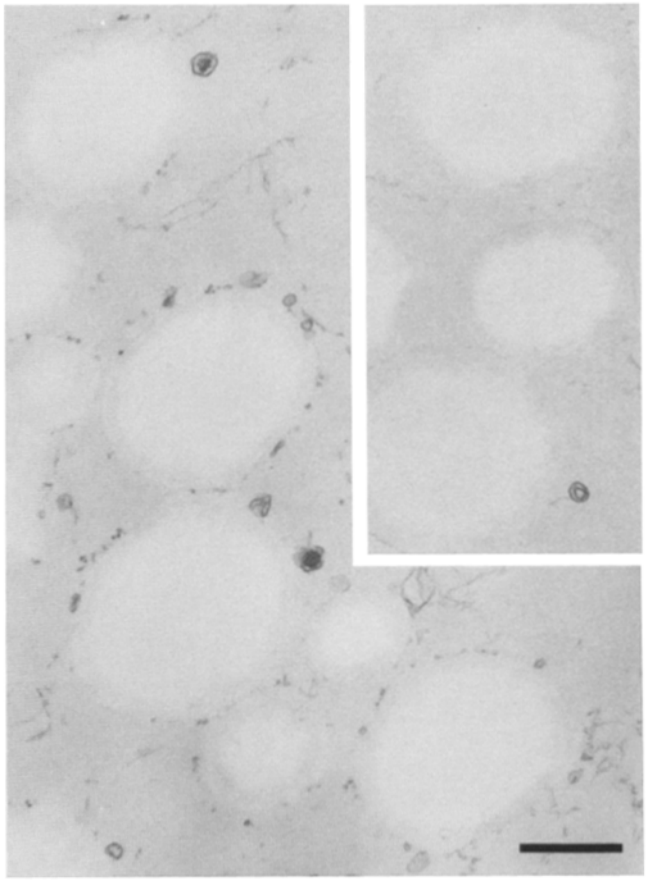

Figure 10. Electron micrographs illustrating organelles which bind to anti-synaptobrevin 2 immunobeads. Small pleomorphic vesicles as well as dense-core granules are visible on the anti-synaptobrevin 2 immunobeads. Electron-dense filamentous material is represented by the agarose used for bead embedding. The inset shows lack of organelle binding to immunobeads conjugated to control mouse IgGs. Bar, 400 nm.

three v-SNAREs of the synaptobrevin family in the membranes of dense-core granules (Fig. 10), supports the hypothesis that this inhibitory effect may be mediated by cleavage of these proteins. We investigated therefore whether inhibition of catecholamine release correlates with a parallel cleavage of the three proteins. As shown in Fig. 10, TeTx-LC produced a strong inhibition of Ca^{2+}-evoked [^{3}H]NE release (~50%) which was already maximal at 5 min. It also produced a massive, although not complete, cleavage of the three proteins which was also already maximal at 5 min (Fig. 11). The close temporal correlation between the two effects strongly supports a direct relationship between proteolysis of the synaptobrevins and cellubrevin and the inhibition of secretion. Note that TeTx-LC cleaved not only synaptobrevin 2 and cellubrevin, but also synaptobrevin 1. Although rat synaptobrevin 1 is more resistant to the proteolytic action of this toxin than synaptobrevin 2, cleavage of synaptobrevin 1 by TeTx in synaptic vesicles was previously reported (Schiavo et al., 1993; Yamasaki et al., 1994). In our experiments, no unspecific proteolysis mediated by 300 nM TeTx-LC of synaptic vesicle proteins other than synaptobrevins and cellubrevin was observed (not shown). The inhibition of Ca^{2+}-evoked catecholamine secretion from PC12 cells produced by TeTx was only about 50% in spite of the nearly complete cleavage of synaptobrevins and cellubrevin. Similar incomplete inhibition of secretion in spite of massive cleavage of these proteins of the synaptobrevin/cellubrevin family was observed in other systems (Galli et al., 1994; Gaisano et al., 1994). These findings suggest the possible existence of other v-SNAREs more substantially divergent from synaptobrevin 1 and 2 and cellubrevin.

Synaptobrevin 1 and 2 were previously shown to interact with the neuronal t-SNAREs syntaxin I and SNAP25. To further prove that cellubrevin and the two synaptobrevins are functionally similar, we investigated whether cellubrevin forms a complex with the same two t-SNAREs which are both expressed in PC12 cells. Triton X-100 extracts of PC12 cells postnuclear supernatants were immunoprecipitated with antibodies directed against synaptobrevin 2, syntaxin or SNAP25. As shown by Fig. 12, anti-syntaxin 1 and anti-SNAP25 antibodies coprecipitated not only the partner t-SNARE and synaptobrevin 2, but also cellubrevin. The specificity of the immunoprecipitations was indicated by the absence of synaptotagmin in all pellets and by the absence of cellubrevin in the immunoisolation performed with

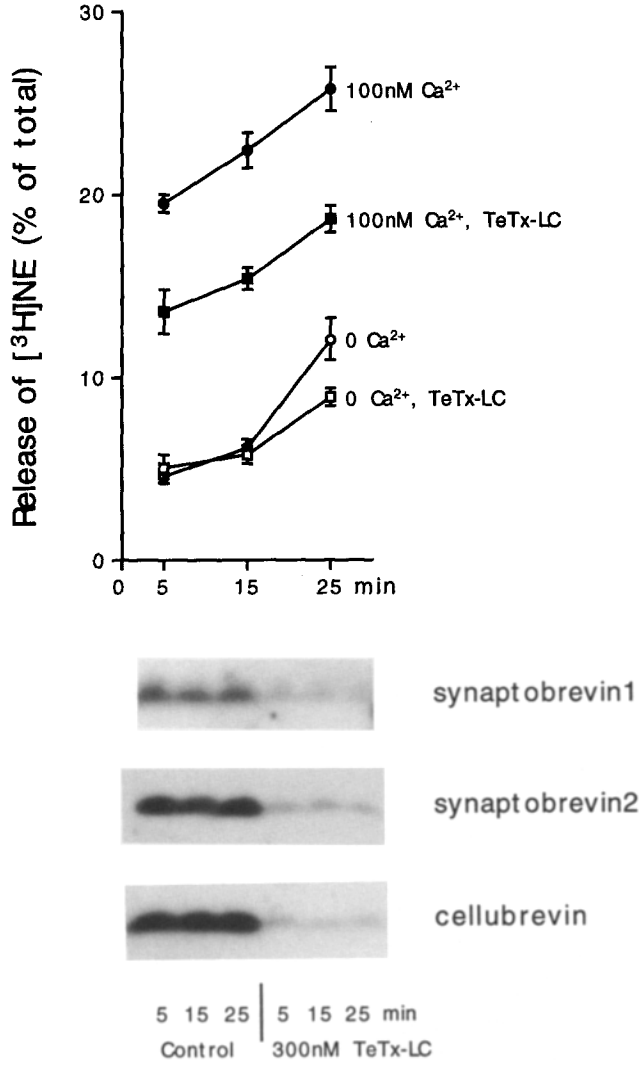

Figure 11. TeTx-LC mediated inhibition of [^{3}H]norepinephrine (NE) release and cleavage of synaptobrevin/cellubrevin in SLO-permeabilized PC12 cells. (*Top*) Fractional release of [^{3}H]NE from cells pre-loaded with the radiolabeled compound. The amount of [^{3}H]NE released in each sample is expressed as the percentage of the total [^{3}H]NE present in that sample (cell-associated plus released [^{3}H]NE). For each time point and treatment, values represent the mean ± SD of three determinations from a representative experiment. (*Bottom*) Synaptobrevin 1 and 2 and cellubrevin content (as determined by Western blotting) of cells used for the determination of [^{3}H]NE release in the absence of Ca^{2+}. Similar cleavage by 300 nM TeTX-LC of synaptobrevin 1 and 2 and cellubrevin was observed in the presence of 100 nM free Ca^{2+} (not shown).

anti-synaptobrevin 2 immunobeads. No v- or t-SNARE were precipitated by non-immune IgGs (not shown).

Discussion

A large body of experimental evidence has indicated that the fundamental mechanisms of exocytosis are very similar in all cells (Aalto et al., 1992; Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994). However, it is clear that additional features must be superimposed on these general mech-

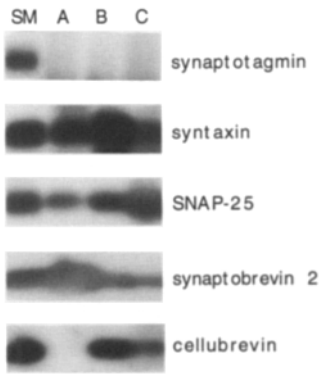

Figure 12. Immunoprecipitation of the SNARE complex. Triton X-100 extract of PC12 cells was immunoprecipitated with antibodies directed against synaptobrevin 2 (*A*), syntaxin 1 (*B*), or SNAP-25 (*C*). 1/10 of the starting material (*SM*), or bead pellets (*A–C*) were analyzed by SDS-PAGE and Western blotting for their content in synaptotagmin 1, syntaxin 1, SNAP-25, synaptobrevin 2, and cellubrevin. Cellubrevin forms complexes with syntaxin 1 and SNAP-25.

anisms to account for specific properties of distinct exocytotic vesicles within the same cell. Synaptobrevin 1 and 2 are v-SNAREs which are primarily expressed in the brain where they are concentrated in synaptic vesicles. These organelles, whose exocytosis is highly regulated, are thought to be a specialized form of endosome-derived vesicles (Cameron et al., 1991; Linstedt and Kelly, 1991). Cellubrevin, a member of the synaptobrevin family, is expressed in a wide variety of tissues and was reported to be localized on endosome-derived, Tf receptor-containing vesicles which undergo exocytosis constitutively (McMahon et al., 1993; Galli et al., 1994). These findings have raised the possibility that the distinct patterns of cellular expression of the three proteins could be related to the property of the two synaptobrevins and cellubrevin to function in distinct secretory pathways. We show here that, when co-expressed physiologically in the same cell, the three proteins have a similar subcellular distribution and interact with the same neuronal t-SNAREs. These results speak against functional differences between synaptobrevin 1, synaptobrevin 2 and cellubrevin related to distinct types of exocytotic vesicles.

To demonstrate whether cellubrevin can be targeted together with synaptobrevins to synaptic vesicles, we have taken advantage of the property of PC12 cells to express all three proteins at relatively high levels. This is in contrast to the pattern of expression of the three proteins in brain, where cellubrevin is primarily localized in non-neuronal cells and is virtually undetectable in neurons. PC12 cells, like other peptide secreting endocrine cells and cell lines, contain a population of small vesicles, synaptic-like microvesicles, which are the functional and structural equivalent of synaptic vesicles of neurons (Navone et al., 1986; Wiedenmann et al., 1988; Cameron et al., 1991; Reetz et al., 1991). Like neuronal synaptic vesicles, synaptic-like microvesicles exclude typical markers of constitutive endosome-derived vesicles such as Tf receptors and LDL receptors (Cameron et al., 1991; Linstedt and Kelly, 1991). Furthermore, endocrine synaptic-like microvesicles are enriched in the neurites that PC12 cells extend after NGF-induced differentiation (Tischler and Greene, 1978). These processes have several other axonal characteristics including the property to exclude Tf receptor (this study) and the G protein of the vesicular stomatitis virus (Rivas and Moore, 1989), two proteins which, in neurons, are selectively targeted to dendrites (Cameron et al., 1991; Dotti and Simons, 1990). Using immuno-

fluorescence and subcellular fractionation, we have demonstrated that cellubrevin and the synaptobrevins have a very similar subcellular distribution in PC12 cells and are colocalized on synaptic-like microvesicles: they are coenriched in neurites, are primarily localized in light fractions of sucrose density gradient which contain synaptic-like microvesicles (Cameron et al., 1991), and are detectable in the synaptic-like microvesicle peak in glycerol velocity gradients. This peak is completely devoid of Tf receptor (this study and Cameron et al., 1991). Thus, cellubrevin does not appear to be a specialized v-SNARE for constitutive recycling Tf receptor containing vesicles.

While the vast majority of the synaptobrevin/cellubrevin molecules in PC12 cells are localized on organelles of low buoyant density, our data are also consistent with a colocalization of a small pool of the three v-SNAREs in the membrane of dense-core secretory granules which store and secrete peptides and amines (Bauerfeind et al., 1993). In previous studies, synaptobrevin immunoreactivity on dense-core granules of neurons and endocrine cells had not been demonstrated (Baumert et al., 1989; Thomas-Reetz et al., 1993). This finding appeared at odds with the demonstration that TeTx-LC inhibits catecholamine secretion from PC12 cells (Ahnert-Hilger and Weller, 1993). As shown in this study, TeTx-mediated cleavage of synaptobrevin/cellubrevin correlates with an impairment of exocytosis of dense-core granules. Thus, although only a small pool of synaptobrevin is localized in the granule fraction, all granule membranes must contain at least a few synaptobrevin/cellubrevin molecules. The existence of at least low levels of these proteins on the membrane of dense core vesicles is suggested by two independent lines of investigation. First, immunoisolation experiments with anti-synaptobrevin 2 antibodies demonstrated that dense-core granules can be quantitatively recovered in the bead fraction. Second, in sucrose density gradients a trail of the synaptobrevin/cellubrevin peaks were observed in regions of high buoyant density which contain secretory granules. When a low-speed supernatant of bovine chromaffin cells, which contains a greater amount of secretory granules, was separated in the same type of gradients, the relative proportion of synaptobrevin found in the dense-granules fraction was greater than in PC12 cells (our unpublished observations).

It appears therefore that synaptobrevin 1 and 2 and cellubrevin share a similar function and may be present in different combinations on the same vesicular carriers as was previously shown for other synaptic vesicle-associated proteins (Sudhof et al., 1989; Geppert et al., 1991; Fykse et al., 1993; Bajjalieh et al., 1993). Isoform specific characteristics may contribute to the fine tuning of the function of exocytotic carriers but do not appear to contribute major functional differences. This is in agreement with studies performed in yeast. At least two synaptobrevin isoforms were detected on yeast secretory vesicles but they appear to have a similar function since one of the two is sufficient to sustain exocytosis and growth (Protopopov et al., 1993). Whether synaptobrevins and cellubrevin play a role also in the exocytosis of constitutive, Golgi-derived vesicles which deliver synaptic vesicle precursor membrane to the cell surface (Regnier-Vigouroux et al., 1991) remains to be elucidated by future studies.

If synaptobrevins and cellubrevin have similar functions in exocytosis, can synaptobrevin 1 and 2 be localized on constitutive recycling vesicles in non-neuronal cells and neuronal cells? The expression of low levels of synaptobrevins in cells lacking classical Ca^{2+}-dependent exocytosis such as muscle cells (Ralston et al., 1994), and adipocytes (Cain et al., 1992) supports this possibility. Furthermore, significant levels of synaptobrevin immunoreactivity are present in the perikaryal dendritic region of immature neurons in culture (Mundigl et al., 1993). As neurons mature and form synapses, synaptobrevin immunoreactivity in dendrites falls to almost undetectable levels, but this change may reflect a down regulation of constitutive recycling at the cell surface of dendrites (Mundigl et al., 1993). For example, clathrin and dynamin immunoreactivity are present only at very low levels in dendrites of most adult neurons (Maycox et al., 1992; McPherson et al., 1994; Takei et al., 1995).

Our previous demonstration that cellubrevin is required for exocytosis in cells which do not express the neuronal t-SNAREs (SNAP25 and syntaxin 1) (Galli et al., 1994), implies that cellubrevin must interact with non-neuronal plasmalemma t-SNAREs (Oyler et al., 1991; Bennett et al., 1993). However, we have shown here that cellubrevin can interact with syntaxin 1 and SNAP25, further proving the functional similarity of synaptobrevin 1, 2 and cellubrevin. In conclusion, our data suggest that cellubrevin, synaptobrevin 1 and synaptobrevin 2 do not play specialized roles in constitutive and regulated secretion, respectively, and are not involved in determining the spatial and temporal specificity of exocytotic reaction of different secretory pathways.

We are indebted to Drs. R. Jahn, I. Trowbridge, P. Greengard, and W. Neupert for the generous gift of antibodies and reagents, to Drs. R. Jahn and T. Südhof for discussion, to H. Tan for photography and T. Taylor for performing FACS® analysis.

This work was supported in part by National Institutes of Health grant (PO1-CA46128) and by a grant from the Juvenile Diabetes Foundation.

Received for publication 12 August 1994 and in revised form 9 January 1995.

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