# Synaptic Vesicle Membrane Proteins Interact to Form a Multimeric Complex

**Mark K. Bennett, Nicole Calakos, Thane Kreiner, and Richard H. Scheller**

Department of Molecular and Cellular Physiology, Howard Hughes Medical Institute, Stanford University Medical Center, Stanford, California 94305

*Abstract.* Potential interactions between membrane components of rat brain synaptic vesicles were analyzed by detergent solubilization followed by size fractionation or immunoprecipitation. The behavior of six synaptic vesicle membrane proteins as well as a plasma membrane protein was monitored by Western blotting. Solubilization of synaptic vesicle membranes in CHAPS resulted in the recovery of a large protein complex that included SV2, p65, p38, vesicle-associated membrane protein, and the vacuolar proton pump. Solubilization in octylglucoside resulted in the preservation of interactions between SV2, p38, and rab3A, while solubilization of synaptic vesicles with Triton X-100 resulted in two predominant interactions, one involving p65 and SV2, and the other involving p38 and vesicle-associated membrane protein. The multicomponent complex preserved with CHAPS solubilization was partially reconstituted following octylglucoside solubilization and subsequent dialysis against CHAPS. Reduction of the CHAPS concentration by gel filtration chromatography resulted in increased recovery of the multicomponent complex. Examination of the large complex isolated from CHAPS-solubilized vesicles by negative stain EM revealed structures with multiple globular domains, some of which were specifically labeled with gold-conjugated antibodies directed against p65 and SV2. The protein interactions defined in this report are likely to underlie aspects of neurotransmitter secretion, membrane traffic, and the spatial organization of vesicles within the nerve terminal.

SYNAPTIC transmission is the primary basis of intercellular communication within the nervous system. Central to this process are the synaptic vesicles responsible for the storage and exocytotic release of neurotransmitter. The life cycle of a synaptic vesicle includes steps of biogenesis, exocytosis, and membrane recycling (for reviews see Kelly, 1988; Südhof and Jahn, 1991; Trimble et al., 1991). Synaptic vesicle components are synthesized in the cell body and transported down the axon to the presynaptic nerve terminal. Within the terminal, the vesicles are loaded with neurotransmitter and stored either adjacent to the presynaptic plasma membrane at the active zone, or in clusters associated with cytoskeletal elements. Stimulation of the nerve terminal results in calcium influx which effects fusion of the synaptic vesicles with the plasma membrane. After exocytosis, synaptic vesicle membrane components are selectively recovered from the presynaptic plasma membrane by endocytosis and are recycled within the nerve terminal into mature synaptic vesicles. A molecular description of these membrane trafficking events and their physiological regulation is required for a more detailed understanding of synaptic transmission.

One approach toward understanding the molecular mechanisms involved in synaptic transmission has been to identify and characterize the components of the synaptic vesicle membrane. A number of proteins are localized to synaptic vesicles, including SV2 (Buckley and Kelly, 1985), p65 (synaptotagmin; Matthew et al., 1981), p38 (synaptophysin; Wiedenmann and Franke, 1985; Jahn et al., 1985), vesicle-associated membrane protein (VAMP)[1] (synaptobrevin; Trimble et al., 1988; Baumert et al., 1989), rab3A (smg25A; Matsui et al., 1988; Zahraoui et al., 1989; Fischer von Mollard et al., 1990; Volknandt et al., 1991), the vacuolar proton pump (Chidon and Shira, 1989; Yamagata and Parsons, 1989; Floor et al., 1990), synapsins I and II (DeCamilli et al., 1983; Südhof et al., 1989a), a high molecular weight proteoglycan (Stadler and Dowe, 1982; Carlson and Kelly, 1983), VAT-1 (Linial et al., 1989), and p29 (Baumert et al., 1990). Many of these proteins are evolutionarily conserved and are localized to vesicle populations containing different classes of neurotransmitter, suggesting that they play a fundamental role in the targetting, fusion, and recycling of synaptic vesicle membranes. An understanding of the functions of these proteins requires both determination of the properties of each protein individually, and characterization of their interactions with other proteins. The isolation of cDNA clones encoding several synaptic vesicle membrane proteins has greatly increased our knowledge about the individual proteins and has suggested possible functions (see Südhof

1. *Abbreviation used in this paper*: VAMP, vesicle-associated membrane protein.

and Jahn, 1991). However, very little is known about interactions that involve these proteins.

The present study was undertaken to identify and characterize the potential interactions among some of the known synaptic vesicle proteins. We have concentrated our analysis on the six membrane proteins diagrammed in Fig. 1 and described below. (*a*) SV2 (75–150 kD) is a glycosylated integral membrane protein recognized by a mAb (Buckley and Kelly, 1985) with an as yet unknown structure or function. (*b*) p65 (65 kD) is an abundant synaptic vesicle protein with a single transmembrane domain, a $NH_2$-terminal intravesicular domain, and a cytoplasmic domain containing two repeats homologous to the regulatory domain of protein kinase C (Perin et al., 1990; Wendland et al., 1991). This protein binds phospholipids, has hemagglutination activity, and has recently been shown to exist in a multimeric complex (Perin et al., 1990, 1991). It has been proposed that p65 plays a role in the docking and/or fusion of synaptic vesicles with the plasma membrane. (*c*) p38 (40 kD) is an abundant vesicle glycoprotein that has four transmembrane domains (Buckley et al., 1987; Leube et al., 1987; Südhof et al., 1987; Cowan et al., 1990), binds $Ca^{2+}$ (Rehm et al., 1986), and has the capacity to form homooligomeric structures that can generate channels in artificial bilayers (Rehm et al., 1986; Thomas et al., 1988; Johnston and Südhof, 1990). It has been proposed that p38 might function in the formation of fusion pores during exocytosis. (*d*) VAMP is an 18-kD protein anchored to the synaptic vesicle membrane by a COOH-terminal hydrophobic domain (Trimble et al., 1988; Südhof et al., 1989b). The function of VAMP is unknown, but recently several proteins with domain structures similar to VAMP have been identified in yeast (Dascher et al., 1991; Shim et al., 1991). These proteins have been shown in genetic studies to interact with a number of proteins required for a functional secretory pathway, including the low molecular weight GTP-binding protein YPT1. (*e*) rab3A (25 kD) is a member of a family of low molecular weight GTP-binding proteins that have been implicated in the regulation of intracellular membrane traffic (Zahraoui et al., 1989; Salminen and Novick, 1988; Segev et al., 1988; Chavrier et al., 1990; Gorvel et al., 1991). As with other low molecular weight GTP-binding proteins, rab3A is likely to be localized to membranes, at least in part, by a COOH-terminal isoprenoid group (Hancock et al., 1989; Johnston, et al., 1991). (*f*) The vacuolar proton pump is a large multimeric complex (500 kD) made up of at least seven subunits (Nelson, 1989; Stone et al., 1989). Subunits A and B form the catalytic ATP-binding domain of the pump, while subunit c is a proteolipid that forms the proton-conducting pore. The proton pump is localized to a variety of intracellular organelles where it functions in the acidification of lumenal compartments. In synaptic vesicles, the proton gradient is used to drive the uptake of neurotransmitters (Chidon and Shira, 1989; Yamagata and Parsons, 1989; Maycox et al., 1990).

We have used detergent solubilization of rat brain synaptic vesicles followed by size fractionation or immunoprecipitation to analyze the interactions between these synaptic vesicle membrane proteins. The distribution of each of the vesicle proteins was evaluated by Western blotting. In the case of the vacuolar proton pump, the 39-kD accessory protein ($H^+$ 39 kD) was monitored. We have found that a large complex including five of the six vesicle proteins is preferentially

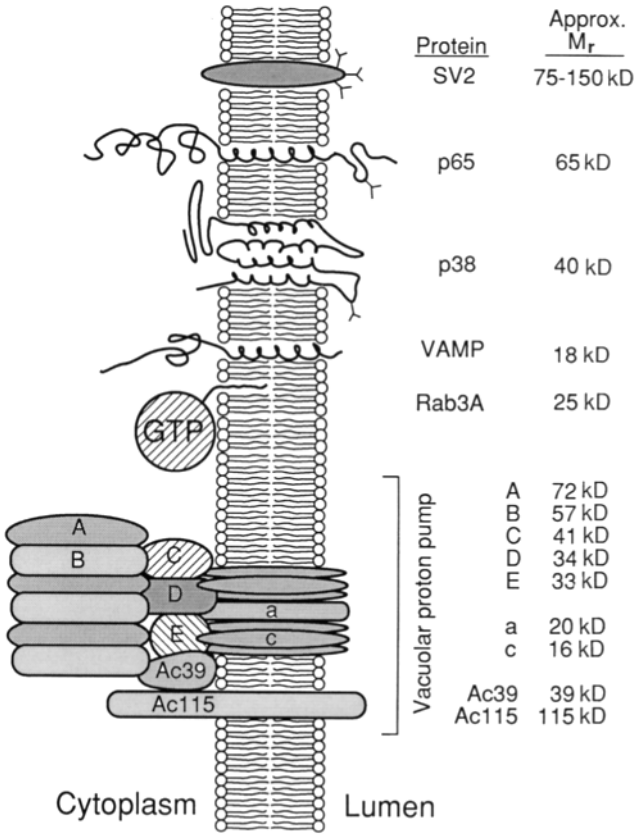


*Figure 1.* Synaptic vesicle membrane proteins. A schematic representation of the synaptic vesicle membrane proteins analyzed in the present study. The structure and subunit nomenclature of the vacuolar proton pump is adapted from Nelson (1989).

preserved following solubilization in CHAPS. In addition, interactions involving subgroups of the vesicle proteins are detected following solubilization in octylglucoside or Triton X-100. These interactions may reflect functional associations of the synaptic vesicle proteins involved in the biogenesis, exocytosis, or recycling of synaptic vesicles.

## Materials and Methods

### Materials

Electrophoresis-grade reagents, gelatin, Triton X-100, and Bio-Gel A-5m column resin were purchased from Bio-Rad Laboratories (Richmond, CA). CHAPS, octylglucoside, and mAb SY38 (anti-p38; Weidenmann and Franke, 1985) were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Anti-mouse IgG, BSA, lipid standards, and reagent grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). $^{125}$I-labeled anti-mouse and anti-rabbit IgG were obtained from ICN Radiochemicals (Irvine, CA). Prestained PAGE molecular weight standards were purchased from Bethesda Research Laboratories (Gaithersburg, MD) and protein A-Sepharose was obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). All centrifuge rotors were from Beckman Instruments (Palo Alto, CA). The following sources of antibodies are gratefully acknowledged. mAb 10H3 (anti-SV2; Buckley and Kelly, 1985) was provided by Dr. Regis Kelly (University of California, San Francisco, CA). mAb M48 (anti-p65; Matthew et al., 1981) was provided by Dr. Louis Reichardt (University of California, San Francisco, CA). Affinity purified ascites fluid for both anti-SV2 and anti-p65 were prepared by Berkeley Antibody Co. (Richmond, CA). Rabbit polyclonal antibodies generated against the 115- and 39-kD accessory proteins of the vacuolar proton pump (Moriyama and Nelson, 1989) were kindly provided by Dr. Nathan Nelson (Roche Institute of Molecular Biology, Nutley, NJ). A rabbit polyclonal antibody generated

against the $\alpha$ subunit of the $Na^+/K^+$ ATPase was provided by Dr. James Nelson (Stanford University, Stanford, CA). Antibodies provided by members of this laboratory were rabbit polyclonal antisera generated against a VAMP fusion protein (Trimble et al., 1990) and against a synthetic peptide corresponding to the conserved hydrophilic domain of VAMP I (peptide B) by Dr. William Trimble, an affinity purified rabbit polyclonal antibody generated against a COOH-terminal peptide of rab3A by Dr. Lisa Elferink (Stanford University), and mAb 6F2 (hybridoma culture supernatant) directed against *Aplysia* atrial gland dense core granules prepared by Dr. Wayne Sossin (Stanford University).

## Synaptic Vesicle Preparation

A synaptic vesicle fraction was prepared by a modification of the procedure described by Huttner et al. (1983). All procedures were carried out at 4°C with ice-cold buffers. Brains from 150 g male Sprague Dawley rats were homogenized in 0.32 M sucrose, 10 mM Hepes-KOH (pH 7.5), 1 mM EGTA, 0.1 mM EDTA, 0.3 mM PMSF (10 ml/brain) by eight up-and-down strokes with a Teflon/glass homogenizer. A postnuclear supernatant was prepared by centrifugation of the homogenate at 2,500 rpm (1,000 *g*) for 10 min in a JS-13 rotor. A synaptosomal pellet was obtained by centrifugation of the postnuclear supernatant at 12,500 rpm (25,000 *g*) for 13 min in the same rotor. The synaptosomal pellet was washed once by resuspension in Hepes-buffered saline (142 mM NaCl, 2.4 mM KCl, 1 mM $MgCl_2$, 5 mM D-glucose, 0.1 mM EGTA, 10 mM Hepes-KOH (pH 7.5), 0.3 mM PMSF; 8 ml/brain) and recentrifugation, resuspended in Hepes-buffered saline (1.6 ml/brain), and then lysed by dilution into 10 volumes of $H_2O$. The lysis and release of synaptic vesicles was promoted by homogenization with a Teflon/glass homogenizer (five up-and-down strokes) and incubation on ice for 30 min. Large membrane fragments were removed by centrifugation at 15,000 rpm (42,000 *g*) for 16 min in an SW28 rotor and synaptic vesicles (and small membrane fragments) were recovered by centrifugation of the resulting supernatant at 28,000 rpm (141,000 *g*) for 4 h in an SW28 rotor. The resulting pellet (LP2) was used as a crude synaptic vesicle fraction or, in some cases, purified further by size exclusion chromatography on a controlled pore glass bead column.

## Sucrose Gradient Sedimentation

An LP2 fraction prepared from five brains was resuspended in 4.5 ml HKA buffer (10 mM Hepes-KOH (pH 7.5), 140 mM potassium acetate, 1 mM $MgCl_2$, 0.1 mM EGTA), and divided into nine 250-$\mu$l fractions (0.5 mg protein each). The fractions were solubilized by addition of an equal volume of one of three detergent solutions (4% CHAPS, 8% octylglucoside, or 4% Triton X-100 prepared in HKA buffer; three fractions with each detergent) followed by end-over-end mixing at 4°C for 1 h. Insoluble material was removed by centrifugation at 15,000 rpm (18,500 *g*) for 15 min in a refrigerated microfuge. Greater than 90% of the synaptic vesicle markers were recovered in the supernatant under all three solubilization conditions (not shown). The solubilized vesicle fractions were layered onto nine 4.5-ml linear 5–20% sucrose gradients (prepared in HKA buffer containing 1% CHAPS, 1% octylglucoside, or 0.1% Triton X-100; three gradients with each detergent) in SW55 centrifuge tubes and subjected to centrifugation at 50,000 rpm (300,000 *g*) for 1.5, 5, or 16 h (one gradient of each detergent for each time). 10 0.5-ml fractions were collected starting from the top of each gradient with an Auto Densi-flow IIC gradient fractionator (Buchler Instruments, Lenexa, KS). The protein in each fraction was concentrated by TCA precipitation and solubilized in 100 $\mu$l SDS gel sample buffer. The pellets in the SW55 tubes following gradient fractionation were solubilized directly in 100 $\mu$l SDS gel sample buffer.

## Immunoprecipitations

Five samples of protein A-Sepharose (50 $\mu$l packed beads) were each suspended in 1 ml HKA buffer containing 0.1% gelatin and 0.1% BSA and the following antibody preparations added: (*a*) 80 $\mu$l hybridoma 6F2 culture supernatant; (*b*) 4 $\mu$l anti-p65 ascites fluid; (*c*) 50 $\mu$l anti-SV2 affinity purified ascites fluid (0.78 mg/ml); (*d*) 10 $\mu$l rabbit anti-mouse IgG (2.2 mg/ml); and (*e*) 20 $\mu$l anti-VAMP (fusion protein) polyclonal serum. The antibody/bead mixture was incubated with end-over-end mixing for 2 h at 4°C and then washed three times with HKA buffer containing 0.1% gelatin. After the last wash, each sample was divided into four equal aliquots equivalent to 12.5 $\mu$l packed beads. Solubilized vesicle samples (prepared as described below) were added to three of the bead samples and the fourth was solubilized directly in 60 $\mu$l SDS sample buffer. While the antibodies were binding to the protein A-Sepharose, an LP2 fraction prepared from five brains was resuspended in 10 ml HKA buffer (1 mg of protein/ml) and divided into three 2.5-ml aliquots. The samples were solubilized by addition of CHAPS, octylglucoside, or Triton X-100 to final concentrations of 2, 4, and 2%, respectively, followed by end-over-end mixing for 1 h at 4°C. Insoluble material was removed by centrifugation (18,500 *g* for 15 min) and gelatin and BSA were each added to a concentration of 0.1%. Four 500 $\mu$l aliquots of the solubilized vesicles were added to the antibody/protein A-Sepharose complexes prepared as described above (bead samples a, b, c, and e). A fifth 500-$\mu$l aliquot of the solubilized vesicles was incubated with 1.5 $\mu$g anti-p38 for 2 h before addition to bead samples d. Binding was allowed to occur for 2 h at 4°C with end-over-end mixing after which time the beads were recovered by brief centrifugation in a microfuge. The beads were washed three times with 1 ml HKA buffer containing 0.1% gelatin and 2% detergent (the same as was used for solubilization). Immunoprecipitated proteins were recovered by addition of 60 $\mu$l SDS gel sample buffer to each sample of beads.

## Lipid Analysis

Lipid extractions were performed on LP2 (100 $\mu$l, 2 mg protein/ml), intact vesicles immunoprecipitated with anti-p65 from 1 ml 0.5 M NaCl-treated LP2, and the multimeric complex immunoprecipitated with anti-p65 from 1 ml CHAPS-solubilized LP2. Each sample was mixed with 600 $\mu$l $CHCl_3$:methanol:HCl (2/1/0.02) followed by 400 $\mu$l methanol:0.2 M KCl (1/1) and the phases separated by brief centrifugation in a microfuge. The organic phase was collected, dried under $N_2$, and resuspended in 20 $\mu$l $CHCl_3$:methanol (95/5). The extracted lipids were spotted on silica gel 60 TLC plates (Merck, Darmstadt, Germany), developed in $CHCl_3$:methanol:acetic acid (65/15/10), and detected with 10% $CuSO_4$/8% $H_3PO4$ and baking at 180°C for 10 min (Yao and Poduslo, 1988). The protein remaining in the interface and aqueous phase following extraction was recovered by TCA precipitation and suspended in 100 $\mu$l SDS gel sample buffer.

## Reconstitution of the Vesicle Protein Complex

A 2-ml sample of LP2 (1 mg of protein/ml in HKA buffer) was solubilized by addition of octylglucoside to a concentration of 4% and mixing for 1 h. The octylglucoside was exchanged for CHAPS by dialysis against 250 ml HKA buffer containing 0.5% CHAPS for 13 h with one change of buffer. The dialysate was brought to a concentration of 2% CHAPS and insoluble material removed by centrifugation (18,500 *g* for 15 min). 1/2-ml aliquots were used for immunoprecipitation with control beads (6F2) or anti-p65 beads as described above.

## Gel Filtration Chromatography

A 0.5-ml sample of LP2 (2 mg protein/ml in HKA buffer) was solubilized by addition of 0.5 ml HKA buffer containing 4% CHAPS followed by end-over-end mixing for 1 h. Insoluble material was removed by centrifugation (18,500 *g* for 15 min) and the resulting supernatant was loaded onto a 1.0 × 49 cm Bio-Gel A-5m column equilibrated with HKA buffer containing 1% CHAPS. 1-ml fractions were collected and proteins recovered by TCA precipitation (total protein) or immunoprecipitation (protein complexes) as described in the text. In a separate experiment, an LP2 fraction from 10 brains was further enriched by controlled pore glass bead chromatography. The included vesicle peak fractions were pooled, concentrated by ultracentrifugation, and solubilized in 4.5 ml HKA containing 2% CHAPS (0.22 mg protein/ml). After removal of insoluble material, the sample was loaded onto a 2.5 × 50 cm Bio-Gel A-5m column equilibrated with HKA buffer containing 0.25% CHAPS. 5-ml fractions were collected and the proteins from 0.5-ml aliquots of each were concentrated by TCA precipitation.

## Preparation of Anti-p65 and Anti-SV2 Gold Conjugates

8-nm colloidal gold was prepared using a modification of the method of Muhlpfordt (1982). Briefly, 1 ml of 1% $HAuCl_4$ was added to 99 ml distilled, deionized $H_2O$ in a 500 ml acid-cleaned Erlenmeyer flask, brought to a slow boil, and boiled for 5 min. A premixed sterile filtered solution of 2.0 ml 1% sodium citrate and 0.50 ml 1% tannic acid was added rapidly with swirling, and the mixture boiled for 30 min. The gold sol was cooled and stored for 1 wk at 4°C in 50-ml conical tubes. Affinity purified anti-p65 (1.5 mg/ml) was dialyzed overnight against 0.1 M sodium borate, pH 8.6, with three changes of buffer. The colloidal gold sol was titrated against the dialyzed antibody; 47.5 ml of the sol were stabilized with 210 $\mu$l of antibody. BSA was added to a final concentration of 0.25% from a sterile, filtered 10%

stock solution. 50 ml Auro-beads (20 nm; Amersham, Arlington Heights, IL) were coupled to 380 μl affinity purified anti-SV2 (0.78 mg/ml) essentially as described above. The gold-conjugated antibodies were subjected to centrifugation for 75 min at 20,000 rpm (70,000 *g*) in an SW28 rotor. The soft pellets were recovered and dialyzed overnight in PBS/glycerol (1/1), and thereafter stored at −20°C.

### Negative Staining

Four aliquots of synaptic vesicles (isolated by control pore glass bead chromatography; 1 mg protein) were each solubilized in 1 ml HKA buffer containing 2% CHAPS for 1 h at 4°C followed by removal of insoluble material by centrifugation (18,500 *g* for 15 min). The first sample (*a*) was directly loaded onto a 1.0 × 49 cm BioGel A-5m gel filtration column equilibrated with HKA buffer containing 1% CHAPS. The second sample (*b*) was incubated with 10 μl gold-conjugated anti-p65 for 2 h at 4°C before loading onto the gel filtration column. The third sample (*c*) was incubated with 5 μl gold-conjugated anti-SV2 and 10 μl gold-conjugated anti-p65 for 2 h at 4°C before loading onto the gel filtration column. The final sample (*d*) was incubated with 100 μl affinity purified anti-p65 (1.5 mg/ml) for 30 min before addition of 10 μl of gold-conjugated anti-p65. The incubation was continued for 1.5 h at which time the mixture was loaded onto the gel filtration column. Fractions 18, 19, and 20 corresponding to the elution position of the multicomponent complex (see Results) were pooled. Aliquots (50 μl) of pooled fractions were fixed by adding an equal volume of 2× fixative consisting of 4% glutaraldehyde, 2% paraformaldehyde, 2% acrolein, and 2 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.2 Glow-discharged formvar-coated 75 mesh nickel grids were floated on droplets of the sample-fixative mixture for 30 min, and then briefly washed in distilled, deionized $H_2O$. The grids were then stained for 20–40 s in 2% aqueous uranyl acetate, pH 3.7, and excess stain removed with filter paper (Hayat, 1989). After drying, the grids were examined at 80 kV in a Philips 410 TEM, equipped with a $LaB_6$ filament.

### Western Blotting

Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose in 384 mM glycine, 50 mM Tris-base, and 20% methanol (Towbin et al., 1979). The nitrocellulose filters were incubated with blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% BSA, 0.5% Tween 20) for 30 min and then cut into horizontal strips based on the migration of prestained molecular weight markers flanking the samples to be analyzed. The region of the blot from the 68-kD marker to the top was probed with either anti-SV2 (1:1,000), anti-Na/K ATPase (1:1,000), or antibody to the 115-kD accessory protein of the proton pump (1:200). The region of the blot between 43 and 68 kD was probed with anti-p65 (1:20,000). The strip between 29 and 43 kD was probed with either anti-p38 (50 ng/ml) or anti-$H^+$ 39 kD (1:200). The region of the blot between 18 and 29 kD was probed with anti-rab3A (1:100), and that between 14 and 25 kD was probed with anti-VAMP (anti-peptide B, 1:1,000). Incubation with the primary antibody was carried out for 2 to 16 h. The blots were washed three times for 5 min with blocking buffer and then incubated with $^{125}I$-labeled anti-mouse or anti-rabbit secondary (1–2 μCi/ml in blocking buffer) for 2 h. The blots were again washed three times for 5 min each, blotted dry, and exposed to XAR-5 film in the presence of an intensifying screen. Exposure times varied between 2 h and 2 d.

### Other Methods

Proteins were concentrated for PAGE by addition of TCA to a final concentration of 20%. The TCA-precipitated protein was recovered by centrifugation at 15,000 rpm (18,500 *g*) for 10 min in a refrigerated microfuge, washed once with ice cold acetone to remove excess TCA, and solubilized in SDS sample buffer. SDS-PAGE was performed by the method of Laemmli (1970). The stacking gel (10 × 90 × 1.5 mm) contained 4.8% acrylamide/0.2% bisacrylamide and the resolving gel (55 × 90 × 1.5 mm) contained 12% acrylamide/0.2% bisacrylamide. SDS-PAGE sample volumes were 20 μl. Silver staining of SDS gels was carried out as described by Heukeshoven and Dernick (1985). Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL; Smith et al., 1985). Quantitation of stained gels and autoradiograms was performed with a computing densitometer (Molecular Dynamics, Sunnyvale, CA). The stoichiometry of the synaptic vesicle proteins was determined by dividing the integrated Coomassie blue staining intensity of each protein by its relative molecular weight. The resulting value was divided by the corresponding value for p65 to obtain a molar ratio relative to p65.

## Results

### Sucrose Gradient Sedimentation

Potential interactions involving synaptic vesicle membrane proteins were analyzed by detergent solubilization and subsequent velocity sedimentation on sucrose density gradients. A synaptic vesicle fraction prepared from rat brain was solubilized in one of three detergents. The three detergents, one zwitterionic (CHAPS) and two nonionic (octylglucoside and Triton X-100), were chosen because of their nondenaturing properties and abilities to preserve functional membrane protein complexes (Hjelmeland and Chrambach, 1984). The solubilized vesicle fractions were resolved on 5–20% sucrose gradients prepared in the corresponding detergent. Three sedimentation times were selected to facilitate analysis of material with a wide range of sedimentation velocities. Fractions from each gradient (including the pellet) were examined by SDS-PAGE and Western blotting for the presence of the following synaptic vesicle markers: SV2, p65, p38, $H^+$ 39 kD, rab3A, and VAMP. The results of such an experiment are presented in Fig. 2. Interactions involving the synaptic vesicle proteins would be expected to result in cosedimentation of two or more markers on a single gradient (Fig. 2, *a–c*) and/or differences in the sedimentation behavior of a single marker among the different detergents (Fig. 2, *d–i*). The relevant observations derived from Fig. 2 have been classified by detergent and are described below.

*CHAPS.* The sedimentation behavior of the CHAPS-solubilized synaptic vesicle proteins is presented in Fig. 2 *a*. The presence of a large protein complex was suggested by the rapid sedimentation of SV2, p65, p38, $H^+$ 39 kD, and VAMP. A portion of each of these proteins migrated well into the gradient after 1.5 h of sedimentation, away from the majority of protein (not shown), and was recovered in the pellet after 5 h of sedimentation. Of the proteins examined, only rab3A was excluded from the complex. This rapidly sedimenting complex was not observed in either octylglucoside- (Fig. 2 *b*) or Triton X-100–solubilized vesicle preparations (Fig. 2 *c*). This difference among the three detergents is exemplified by the distribution of p38 after 1.5 h of sedimentation (Fig. 2 *d*), and of the $H^+$ 39 kD after 5 h of sedimentation (Fig. 2 *f*). Another component of the vacuolar proton pump, the 115-kD accessory subunit, displayed sedimentation properties identical to the $H^+$ 39 kD (not shown), suggesting that the observed differences among the detergents were not the result of disruption of the multimeric proton pump complex.

The sedimentation behavior of the fraction of each synaptic vesicle protein not included in the large complex was also of interest. Cosedimentation of the p65 and SV2 remaining in the gradient (especially the lower band of the SV2 doublet) was apparent at the 5-h timepoint (Fig. 2 *a*). A comparison of the p65 distribution among the three detergents after 5 h of sedimentation (Fig. 2 *e*) revealed that the CHAPS-solubilized p65 sedimented most rapidly, again suggesting that an interaction involving p65, distinct from the large complex, was preferentially preserved by CHAPS. After 16 h of sedimentation of the CHAPS-solubilized vesicles (Fig. 2 *a*), most of the SV2, p65, and $H^+$ 39 kD were recovered in the pellet. The markers remaining in the gradient were resolved as independent species with relative sedimen-

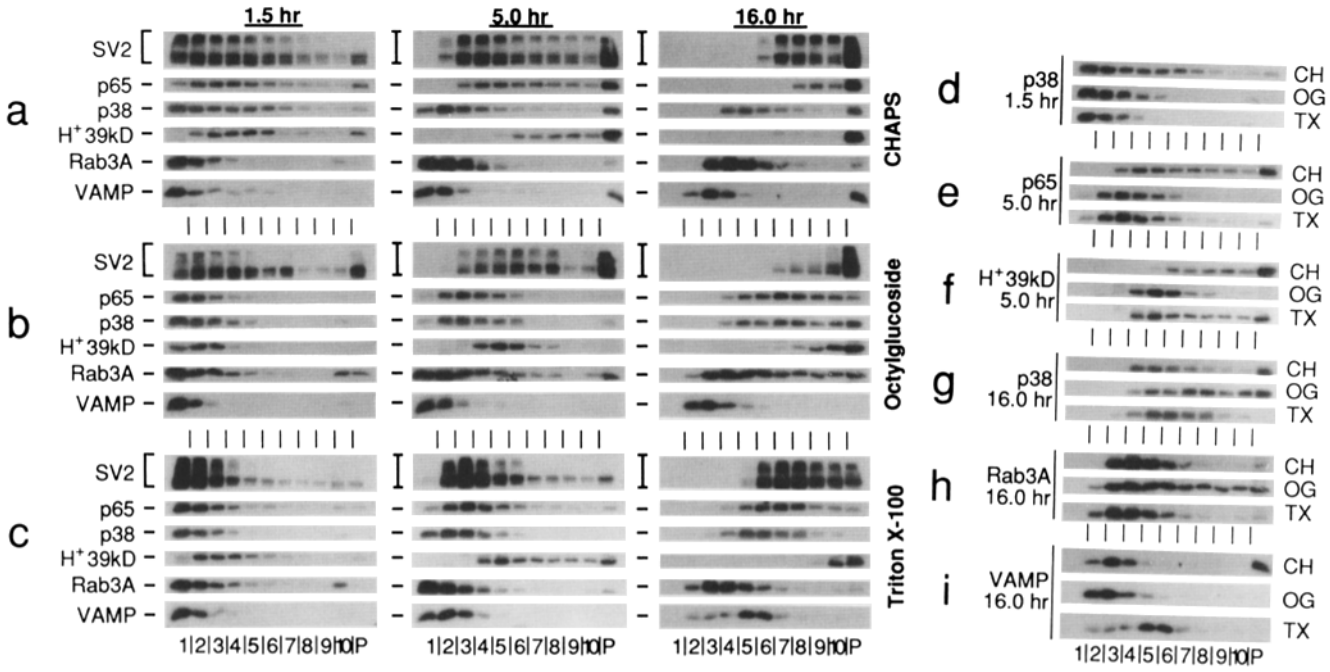


*Figure 2.* Sucrose gradient analysis of detergent-solubilized synaptic vesicles. Synaptic vesicle fractions were solubilized in CHAPS (*a*), octylglucoside (*b*), or Triton X-100 (*c*) and resolved on 5–20% linear sucrose gradients prepared in the corresponding detergent (as described in Materials and Methods). Fractions from the gradients (starting from the top, fraction *1*, through the pellet, *P*) were collected and analyzed by Western blotting for the presence of SV2, p65, p38, $H^+$ 39 kD, rab3A, and VAMP. Panels *d–i* highlight differences among the three detergents for a single marker and timepoint: *d*, p38 at 1.5 h; *e*, p65 at 5 h; *f*, $H^+$ 39 kD at 5 h; *g*, p38 at 16 h; *h*, rab3A at 16 h; and *i*, VAMP at 16 h. The sedimentation behavior of protein standards was determined on parallel gradients. After 1.5 h of sedimentation, apoferritin (17.6S) was recovered in fraction 3 and catalase (11.3S) in fraction 2. After 5 h of sedimentation, catalase was recovered in fraction 5 and ovalbumin (3.5S) in fraction 2. After 16 h of sedimentation ovalbumin was recovered in fraction 5 and cytochrome c (1.75S) in fraction 3.

tation rates of p38 > rab3A > VAMP. The sedimentation of p38 on these gradients was the same as that previously described for a p38 homooligomer (120 kD; Rehm et al., 1986).

***Octylglucoside.*** The sedimentation behavior of the octylglucoside-solubilized synaptic vesicle proteins is presented in Fig. 2 *b*. After 1.5 h of sedimentation only SV2 migrated well into the gradient. By 5 h of sedimentation a large portion of SV2 was recovered in the pellet, and that which remained in the gradient was well resolved from p65, in contrast to the cosedimentation observed with CHAPS-solubilized vesicles. A portion of rab3A, but no other synaptic vesicle marker tested, sedimented as rapidly as SV2 following octylglucoside solubilization (Fig. 2 *b*). Additional differences among the three detergents were observed after 16 h of sedimentation. A portion of both p38 (Fig. 2 *g*) and rab3A (Fig. 2 *h*) sedimented more rapidly in the octylglucoside-solubilized sample than in the CHAPS- or Triton X-100–solubilized samples. This suggests that octylglucoside may at least partially preserve some interactions involving these two proteins.

***Triton X-100.*** The sedimentation behavior of Triton X-100–solubilized synaptic vesicle proteins is presented in Fig. 2 *c*. Co-sedimentation of p65 and SV2 was observed at both the 5- and 16-h timepoints. This co-sedimentation occurred at a slower rate than the cosedimentation of the same two markers in the CHAPS-solubilized sample (Fig. 2 *a*). In addition, cosedimentation of p38 and VAMP was apparent in the Triton X-100–solubilized sample (Fig. 2 *c*). The resulting shift in the distribution of VAMP was most clearly seen after 16 h of sedimentation (Fig. 2 *i*).

The results of the sucrose gradient sedimentation analysis indicate that a number of interactions may occur between different synaptic vesicle proteins, and that these interactions may be differentially preserved by the detergents used in this study. However, several problems exist in the interpretation of these sedimentation results. One problem is that some interactions may be unstable under the conditions of sedimentation: high centrifugal force, high sucrose concentration, and long sedimentation times. These factors could result in partially overlapping sedimentation distributions, or spreading of markers across the gradient as weak interactions are disrupted during sedimentation (Fig. 2). In addition, the sedimentation behavior of membrane proteins is influenced by the amount and type of detergent bound (Tanford and Reynolds, 1976). Some of the sedimentation differences among the three detergents may reflect this fact. Finally, it is difficult on the basis of sedimentation behavior alone to determine if the observed differences in sedimentation result from heterophilic or homophilic interactions, or both. To further define the interactions between synaptic vesicle proteins, we performed immunoprecipitation experiments.

## *Immunoprecipitation*

The results of an immunoprecipitation experiment are presented in Fig. 3. As with the sedimentation analysis, vesi-

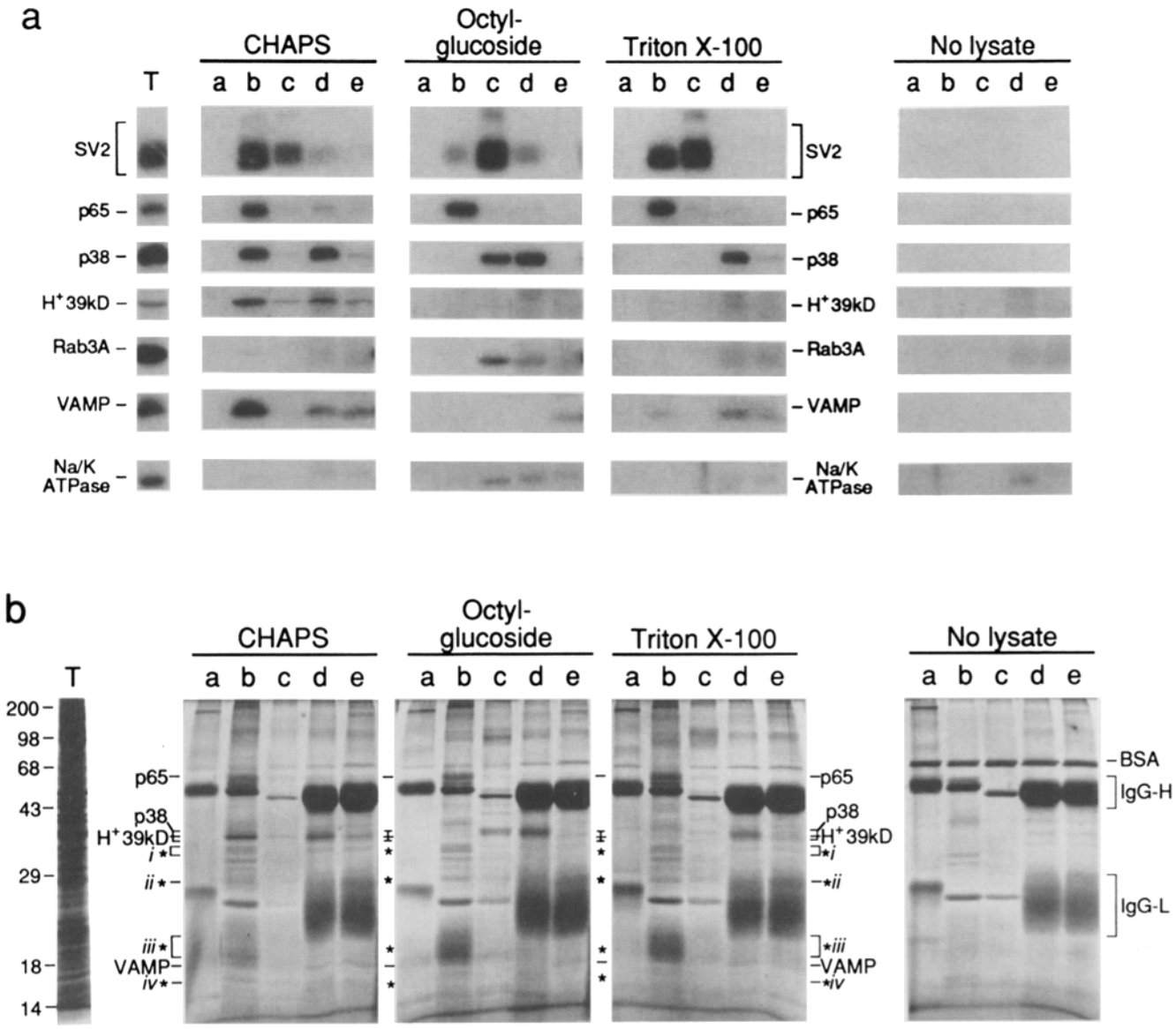


*Figure 3.* Immunoprecipitation analysis of detergent-solubilized synaptic vesicles. Solubilized synaptic vesicle proteins were subjected to immunoprecipitation (as described in Materials and Methods) with the following antibodies: lane *a*, 6F2 (control); lane *b*, anti-p65; lane *c*, anti-SV2; lane *d*, anti-p38; and lane *e*, anti-VAMP. The immunoprecipitates were analyzed by Western blotting for the presence of SV2, p65, p38, $H^+$ 39 kD, rab3A, VAMP, and the $\alpha$ subunit of the $Na^+/K^+$ ATPase (*a*), and by silver staining (*b*). Lane *T* (total) corresponds to 1/10th of the starting sample used for the immunoprecipitations, while the "no lysate" panels display the background contributed by the antibody protein A-Sepharose bead complexes. In *b*, the positions of the molecular weight standards, p65, p38, $H^+$ 39 kD, VAMP, four proteins enriched in the anti-p65 precipitates (*,*i–iv*), BSA, and the IgG heavy and light chains are indicated.

cle fractions were solubilized in CHAPS, octylglucoside, or Triton X-100. Immunoprecipitations were performed using antibodies against four synaptic vesicle proteins (p65, *b* lanes; SV2, *c* lanes; p38, *d* lanes; and VAMP, *e* lanes) as well as a control mAb directed against *Aplysia*-dense core granules (*a* lanes). The immunoprecipitates were resolved on three identical 12% polyacrylamide gels. Two gels were transferred to nitrocellulose for Western blot analysis (Fig. 3 *a*), and the third gel was silver stained to reveal total protein patterns (Fig. 3 *b*). The Western blots were probed for the presence of six synaptic vesicle proteins (SV2, p65, p38, $H^+$ 39 kD, rab3A, and VAMP) as well as a plasma membrane marker (the $\alpha$ subunit of the $Na^+/K^+$ ATPase). The $Na^+/K^+$ ATPase is a contaminant in the vesicle preparation and serves as an internal control for nonspecific aggregation and/or trapping of membrane proteins. A sample of the antibody/bead complex that was not exposed to solubilized synaptic vesicles was also run on the gels to determine the background contributed by the antibody alone ("no lysate" panels). To determine the relative recovery of each marker, a sample of the starting vesicle fraction (corresponding to 10% of the material used for each immunoprecipitation) was also analyzed (lane *T*). The results described below have been divided into observations regarding the background, specific observations concerning each solubilizing detergent, and observations derived from the silver stained gel.

***Background.*** The background signal contributed by antibody alone on Western blots (Fig. 3 *a*, *no lysate*) was restricted to lanes *d* and *e* (where whole rabbit sera were used for the immunoprecipitation). In these lanes a small amount of background on the strips corresponding to $H^+$ 39 kD, rab3A, and $Na^+/K^+$ ATPase was detected. The background contributed by antibody alone to the silver-stained protein pattern (Fig. 3 *b*, "no lysate" panel) consisted of the predominant heavy and light chains of the IgG's as well as several minor bands which varied among the antibody preparations. Under all three detergent conditions used, very little nonspecific binding to the control antibody/protein A-Sepharose complex was detected by either Western blotting (Fig. 3 *a*, *a* lanes) or silver staining (Fig. 3 *b*, *a* lanes).

***CHAPS.*** Antibodies directed against four different synaptic vesicle proteins (p65, SV2, p38, and VAMP) were each capable of immunoprecipitating five synaptic vesicle markers (SV2, p65, p38, $H^+$ 39 kD, and VAMP) from a CHAPS-solubilized vesicle fraction (Fig. 3 *a*, *CHAPS*, lanes *b–e*). A sixth vesicle marker (rab3A) was not precipitated. This result is consistent with the observed cosedimentation of SV2, p65, p38, $H^+$ 39 kD, and VAMP as a large complex in CHAPS-solubilized preparations (Fig. 2 *a*). The immunoprecipitation efficiency of the different markers, by comparison with the starting fraction (Fig. 3 *a*, lane *T*), was variable. For example, anti-p65 (lane *b*) efficiently precipitated p65, SV2, $H^+$ 39 kD, and VAMP, but precipitated p38 with a lower efficiency. Similarly, anti-p38 was efficient at precipitating p38 and VAMP, but relatively inefficient at precipitating SV2 and p65. This variability may reflect the possibility that different fractions of a particular marker are found associated with the large complex. Alternatively, variable immunoprecipitation efficiency may be the result of epitope inaccessibility or destabilization of interactions by antibody binding. In spite of the variable immunoprecipitation efficiency, three lines of evidence indicate that the coprecipitation of multiple markers from CHAPS-solubilized vesicles is specific. First, very little of either the $Na^+/K^+$ ATPase or rab3A present in the starting material was recovered in the immunoprecipitates (Fig. 3 *a*, compare lane *T* with *CHAPS*, lanes *b–e*). Second, immunoprecipitations of octylglucoside- or Triton X-100–solubilized vesicles resulted in coprecipitations of some markers (see below), but not all five markers simultaneously as in CHAPS (Fig. 3 *a*). Finally, the total protein patterns of the immunoprecipitates were much simpler than that of the starting fraction (Fig. 3 *b*), suggesting that nonspecific aggregation or trapping was not occurring.

***Octylglucoside.*** Solubilization of vesicles with octylglucoside followed by immunoprecipitation with anti-p65 (Fig. 3 *a*, *octylglucoside*, lane *b*) resulted in the recovery of p65 and a small amount of SV2 (much less than the recovery in CHAPS or Triton X-100). None of the other immunoprecipitations resulted in significant recovery of p65. Similarly, anti-VAMP (lane *e*) was capable of precipitating only VAMP, and VAMP was not precipitated by any of the other antibodies. These results suggest that p65 and VAMP are, for the most part, independent of the other markers tested. Very different results were obtained with anti-SV2 (lane *c*) and anti-p38 (lane *d*). Both of these antibodies were capable of coprecipitating SV2, p38, and a portion of rab3A from octylglucoside-solubilized vesicles. This observation supports the sedimentation result which suggested that interactions involving rab3A and p38 were preferentially preserved following octylglucoside solubilization (Fig. 2, *g* and *h*). The same arguments concerning specificity made above for the CHAPS-solubilized samples also apply to the octylglucoside-solubilized samples with one notable exception. A significant amount of the $Na^+/K^+$ ATPase was recovered in the anti-SV2 precipitate (Fig. 3 *b*, *octylglucoside*, lane *c*). This was the only antibody/detergent combination that resulted in co-precipitation of the plasma membrane marker. The total protein pattern corresponding to this immunoprecipitate (Fig. 3 *b*, *octylglucoside*, lane *c*) did not display a high background indicative of nonspecific aggregation or trapping. This raises the possibility that the recovery of the $Na^+/K^+$ ATPase under these conditions is specific (see Discussion).

***Triton X-100.*** Solubilization of vesicles with Triton X-100 followed by immunoprecipitation with anti-p65 (Fig. 3 *a*, *Triton X-100*, lane *b*) resulted in efficient recovery of p65 and SV2, and less efficient recovery of $H^+$ 39kD and VAMP. Immunoprecipitation with anti-SV2 (lane *c*) resulted in efficient recovery of SV2 and less efficient recovery of p65. These results support the potential interaction involving p65 and SV2 suggested by their cosedimentation following Triton X-100 solubilization (Fig. 2 *c*). Immunoprecipitation of Triton X-100–solubilized sample with either anti-p38 (lane *d*) or anti-VAMP (lane *e*) resulted in recovery of both p38 and VAMP. This result is again in support of the sedimentation results following Triton X-100 solubilization suggestive of an interaction involving p38 and VAMP (Fig. 2 *c*).

***Silver Staining.*** Several of the synaptic vesicle proteins (p65, p38, $H^+$ 39 kD, and VAMP) were clearly detected in the immunoprecipitates by silver staining (Fig. 3 *b*). In addition to the known synaptic vesicle proteins, several unidentified proteins were recovered in the immunoprecipitates. Most of these proteins were detected only with particular antibody/detergent combinations and often did not correlate with abundant proteins in the starting fraction. Proteins marked with asterisks (Fig. 3 *b*, *i–iv*) were particularly well recovered in anti-p65 precipitates under all three solubilization conditions. Band *i* is a doublet of 35 kD that is not abundant in the starting fraction. Band *ii* (29 kD) co-migrates with an abundant protein in the starting fraction and is the same size as a known synaptic vesicle protein (p29; Baumert et al., 1990). Band *iii* is a heterogeneous protein, or set of proteins, in the 20-kD region that is not abundant in the starting fraction. Band *iv* (16 kD) runs at a position that corresponds to a protein in the starting vesicle fraction that partitions into the organic phase upon chloroform/methanol extraction, and thus may be the proteolipid subunit (subunit *c* in Fig. 1) of the proton pump (Mandel et al., 1988). The nature of these and other coprecipitating proteins will be the topic of future studies.

### *Comparison of the Multimeric Protein Complex with Intact Synaptic Vesicles*

To compare the protein and lipid composition of intact synaptic vesicles with that of the multimeric complex recovered after CHAPS solubilization, anti-p65 immunoprecipitations of salt-washed (intact) and CHAPS-solubilized vesicles were performed. The samples were analyzed for protein composition by SDS/PAGE (Fig. 4 *a*) and for lipid composition by TLC (Fig. 4 *b*). The protein composition of intact vesicles

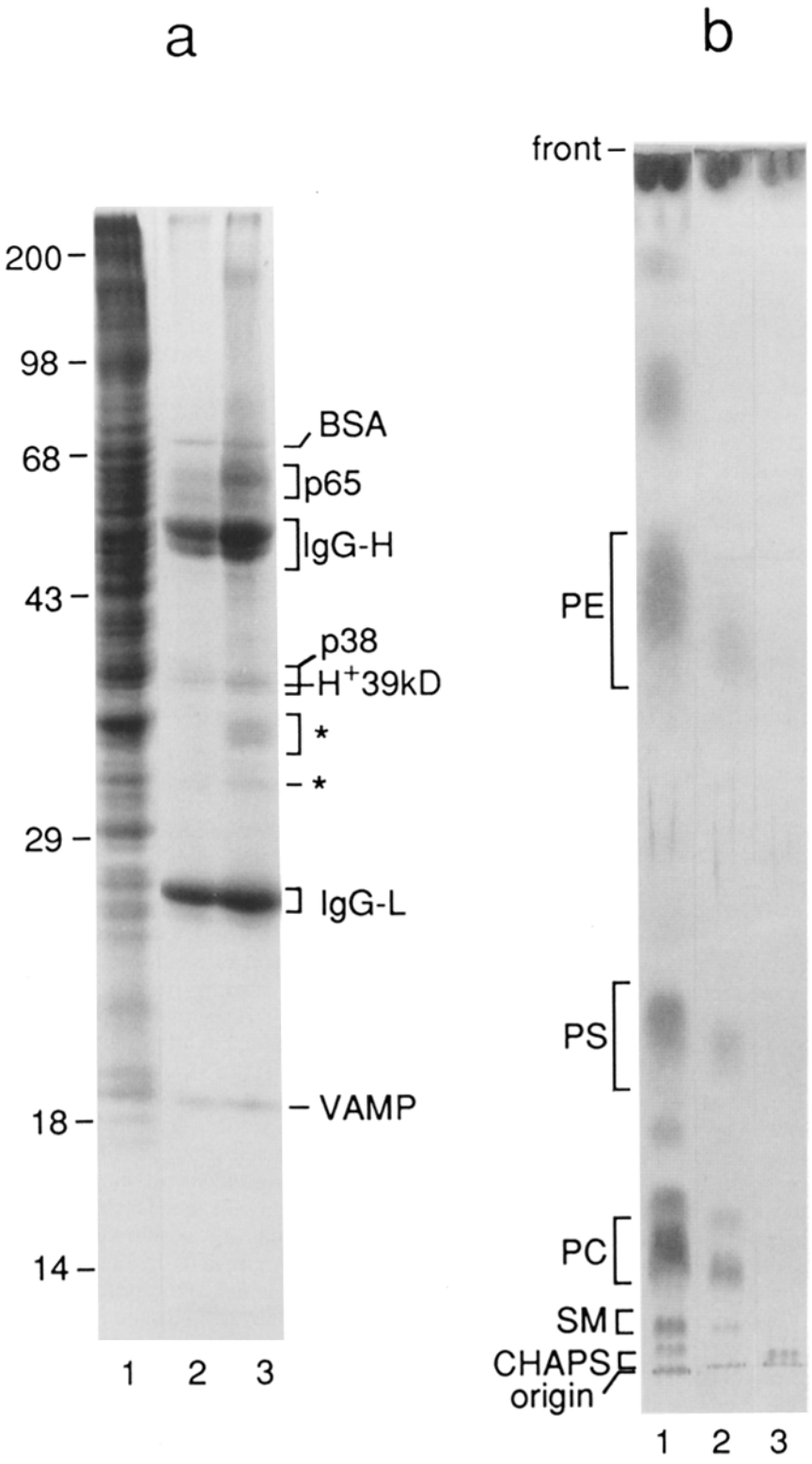


*Figure 4.* Comparison of intact vesicles with the CHAPS-solubilized protein complex. Synaptic vesicles were treated with 0.5 M NaCl or 2% CHAPS and subjected to immunoprecipitation with anti-p65. The starting LP2 fraction (lanes *1*), intact vesicles (lanes *2*), and the CHAPS-solubilized protein complex (lanes *3*) were analyzed for protein composition by SDS-PAGE followed by Coomassie blue staining (*a*) and for lipid composition by TLC (*b*) as described in Materials and Methods. In *a*, the positions of the molecular weight standards, p65, p38, $H^+$ 39 kD, VAMP, two proteins enriched in the CHAPS-solubilized protein complex (*), BSA, and the IgG heavy and light chains are indicated. In *b*, the positions of phosphatidylethanolamine (*PE*), phosphatidylserine (*PS*), phosphatidylcholine (*PC*), sphingomyelin (*SM*), and CHAPS are indicated. The material migrating at the solvent front consisted of nonpolar lipids and an unidentified component derived from the extraction procedure.

(Fig. 4 *a*, lane *2*) was very similar to the multimeric complex (Fig. 4 *a*, lane *3*), and both were highly simplified as compared to the starting fraction (Fig. 4 *a*, lane *1*). The major proteins detected in intact vesicles were p65, p38, $H^+$ 39kD, and VAMP, consistent with published results (Floor and Leeman, 1988; Burger et al., 1989). Peripheral synaptic vesicle membrane proteins (including synapsin I and the catalytic subunits of the proton pump) were not recovered in either the intact vesicles or the multimeric complex. The relative amounts of the known synaptic vesicle membrane proteins were determined by densitometry. The approximate molar ratio of p65:p38:$H^+$ 39 kD:VAMP was 1.0/0.72/0.66/1.6 in intact synaptic vesicles and 1.0/0.13/0.36/0.55 in the CHAPS-solubilized protein complex. This difference in the

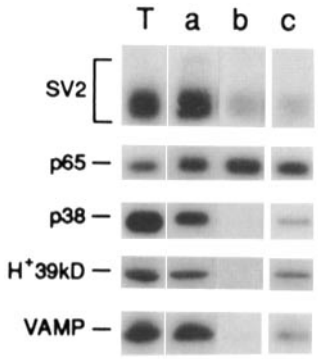


*Figure 5.* Reconstitution of vesicle protein interactions. Synaptic vesicle fractions were solubilized in CHAPS (lane *a*), octylglucoside (lane *b*), or octylglucoside followed by detergent exchange by dialysis against CHAPS (lane *c*) as described in Materials and Methods. Proteins interacting with p65 were detected by immunoprecipitation and Western blot analysis. Lane *T* (total) corresponds to 1/10th of the starting sample used for each solubilization.

ratios indicates that different fractions of each vesicle protein are associated with the multimeric complex. The recovery of p65 was higher in the CHAPS-solubilized protein complex than in the intact vesicles, suggesting that the epitope is more accessible following detergent solubilization. Two unidentified proteins (marked with asterisks) were enriched in the CHAPS-solubilized complex, and may correspond to proteins *i* and *ii* identified in Fig. 3 *b*. The lipid composition of intact synaptic vesicles consisted of predominantly phospholipids (Fig. 4 *b*, lane *2*) and cholesterol (not shown), and lacked several of the lipids detected in the starting fraction (Fig. 4 *b*, lane *1*). The CHAPS-solubilized protein complex contained no detectable phospholipid (Fig. 4 *b*,lane *3*) or nonpolar lipids (not shown) despite high levels of several synaptic vesicle proteins (Fig. 4 *a*). The only spots detectable on the TLC plate were attributable to the presence of CHAPS in the lipid extract. These results demonstrate that the complex of synaptic vesicle proteins recovered after CHAPS solubilization includes a large fraction of the constituent proteins and very little, if any, lipid.

### *Reconstitution of the Vesicle Protein Complex*

The results of both sedimentation and immunoprecipitation experiments on CHAPS-solubilized vesicles suggest the presence of a large complex of synaptic vesicle proteins that includes SV2, p65, p38, $H^+$ 39 kD, and VAMP. To better understand the nature of the interactions involved in this protein complex, a reconstitution experiment was done (Fig. 5). Vesicles were solubilized in octylglucoside and the octylglucoside then exchanged for CHAPS by dialysis. To determine the extent to which interactions that were disrupted by octylglucoside could be restored, the dialyzed sample was subjected to immunoprecipitation with anti-p65 (lane *c*). For comparison, anti-p65 immunoprecipitations were also performed on vesicles directly solubilized in CHAPS (lane *a*) or in octylglucoside (lane *b*). The immunoprecipitates were analyzed by Western blotting for the presence of SV2, p65, p38, $H^+$ 39 kD, and VAMP. As previously observed, anti-p65 precipitated p65, SV2, p38, $H^+$ 39 kD, and VAMP from CHAPS-solubilized vesicles, but only p65 and a reduced amount of SV2 from octylglucoside-solubilized vesicles. After replacement of the octylglucoside with CHAPS, three of the synaptic vesicle proteins (p38, $H^+$ 39 kD, and VAMP) were again recovered by anti-p65 precipitation. SV2 did not reassociate under these conditions. The $Na^+/K^+$ ATPase was not precipitated under any of these conditions (not shown). These results suggest that the interactions among some of the synaptic vesicle proteins can be disrupted and subsequently reconstituted, implying that specific binding or association is involved. Additional experiments (not shown) demonstrated that the vesicle protein complex immunoprecipitated by anti-p65 from a CHAPS-solubilized sample was stable when treated with high salt (1 M potassium acetate), reducing agent (100 mM DTT), $CaCl_2$ (2 mM), or cholate (1%), but was disrupted by Zwittergent 3-10 (2%) or SDS (0.1%).

### *Gel Filtration Chromatography*

To further characterize the synaptic vesicle protein complex observed following CHAPS solubilization, a combined size fractionation and immunoprecipitation analysis was performed. A vesicle fraction solubilized in CHAPS was resolved on a Biogel A-5m gel filtration column equilibrated with 1% CHAPS. Alternate fractions across the column were analyzed by Western blotting (Fig. 6 *a*). A portion of SV2, p65, p38, $H^+$ 39 kD, and VAMP was recovered in a peak just after the void volume (fractions 19-23), again suggestive of a large complex (see also Fig. 7 *b*). The remainders of these markers were resolved over the rest of the column fractions. rab3A eluted as a single well-included peak. The coelution of the five synaptic vesicle proteins near the void volume was not observed with vesicles solubilized in other detergents. In octylglucoside, only SV2 eluted in the region of the void volume; in Triton X-100, none of the markers tested eluted near the void volume (not shown). A portion of the $Na^+/K^+$ ATPase was also recovered in the void region (Fig. 6 *a*), but this behavior was independent of the detergent used for solubilization (not shown). Three separate pools were prepared from different column elution positions (pool I, fractions 18 and 20; pool II, fractions 24 and 26; and pool III, fractions 30 and 32) for subsequent immunoprecipitation analysis (Fig. 6 *b*). Immunoprecipitation of pools I and II with anti-p65 and anti-SV2 resulted in coprecipitation of p65, SV2, p38, $H^+$ 39 kD, and VAMP, suggesting that the multicomponent complex is not exclusively localized to the largest elution position, but rather is spread over a wide range of sizes. Immunoprecipitation of pool III with anti-SV2 resulted in recovery of SV2, p65, and a small amount of VAMP. Immunoprecipitation of pool III with anti-p38 resulted in recovery of p38 and a small amount of VAMP. These results suggest that the material eluting in the later fractions from the gel filtration column is made up of both subcomplexes (SV2-p65 and p38-VAMP) and species that are independent of other markers (rab3A).

The fact that CHAPS-solubilized vesicle proteins can be resolved into complexes with differing sizes and compositions suggests that at least some of the interactions involved in the large multicomponent complex are unstable. In addition, the differential effects of detergents on the vesicle protein interactions and the resistance of the multicomponent complex to high salt suggest that some of the interactions involved are hydrophobic in nature. To determine if the putative weak hydrophobic interactions could be stabilized by reducing the detergent concentration, a vesicle fraction was solubilized in 2% CHAPS and resolved on a Biogel A-5m column equilibrated with 0.25% CHAPS (below the critical micelle concentration of CHAPS). As seen in Fig. 7 *a*, a

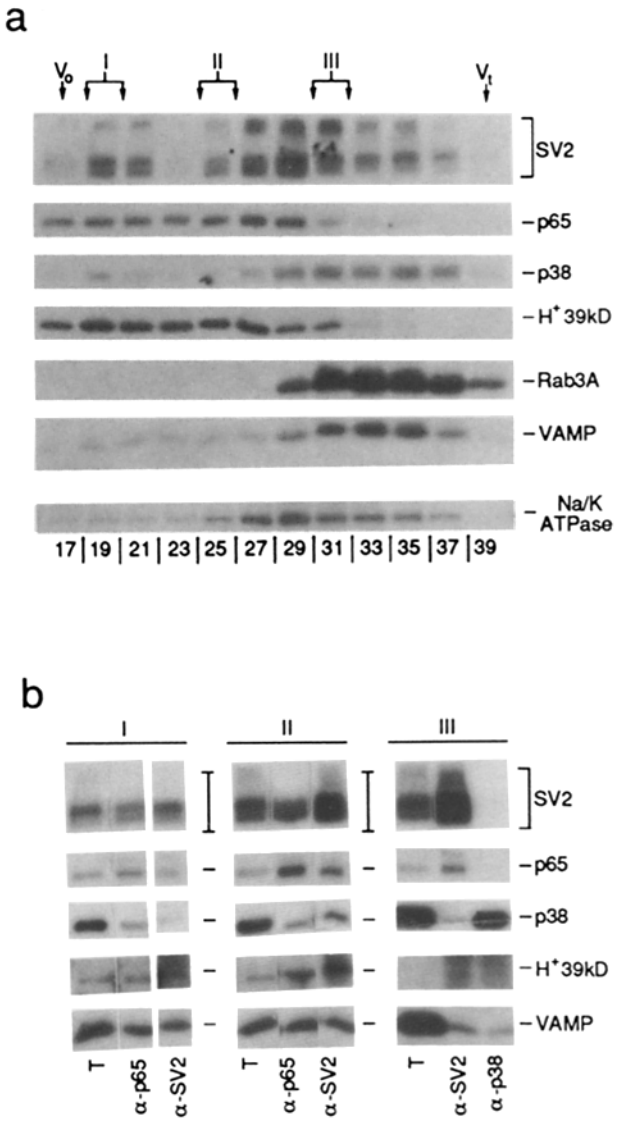


*Figure 6.* Gel filtration analysis of CHAPS-solubilized synaptic vesicles. A synaptic vesicle fraction was solubilized in CHAPS and resolved on a BioGel A-5m column in the presence of 1% CHAPS as described in Materials and Methods. Alternate fractions were analyzed by Western blotting for SV2, p65, p38, $H^+$ 39 kD, rab3A, VAMP, and the $\alpha$ subunit of the $Na^+/K^+$ ATPase (*a*). Three pairs of fractions (marked *I*, *II*, and *III* in *a*) were separately pooled and analyzed by immunoprecipitation and Western blot analysis *b*). $V_o$ and $V_t$ correspond to the void and total volumes of the column, respectively.

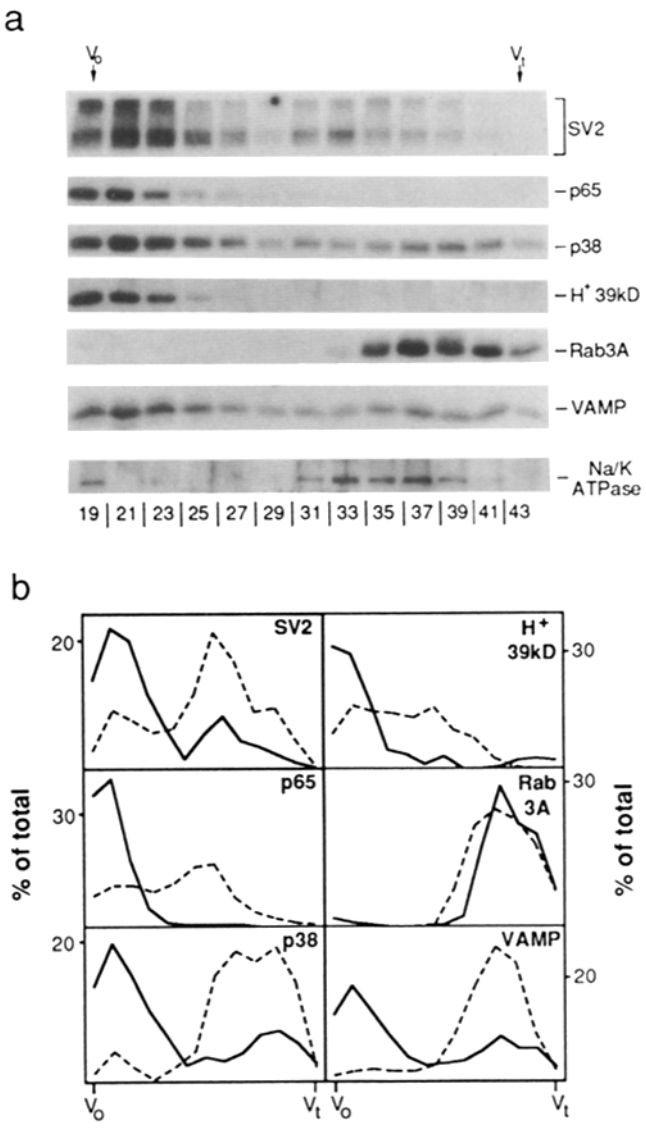


*Figure 7.* Reformation of the large vesicle protein complex by gel filtration. A synaptic vesicle fraction was solubilized in CHAPS and resolved on a BioGel A-5m column in the presence of 0.25% CHAPS. Alternate fractions were analyzed by Western blotting for SV2, p65, p38, $H^+$ 39 kD, rab3A, VAMP, and the $\alpha$ subunit of the $Na^+/K^+$ ATPase (*a*). Quantitation of the column profiles (*b*) for each synaptic vesicle marker in 1% CHAPS (-----, from Fig. 6 *a*) and 0.25% CHAPS (——, from Fig. 7 *a*). $V_o$ and $V_t$ correspond to the void and total volumes of the column, respectively.

striking redistribution of five of the markers occurred when the detergent concentration was lowered. Quantitation of the column profiles of each vesicle marker under high (Fig. 6 *a*) and low (Fig. 7 *a*) CHAPS conditions is presented in Fig. 7 *b*. At low CHAPS concentration, SV2, p65, and $H^+$ 39 kD were recovered almost exclusively in the void region, and much larger fractions of both p38 and VAMP coeluted with this large complex. The distribution of rab3A and $Na^+/K^+$ ATPase remained unchanged, suggesting that the observed differences were not the result of nonspecific aggregation of membrane proteins. In addition, two enzymatic activities associated with synaptic vesicles, an *N*-ethylmaleimide–sensitive ATPase (Yamagata and Parsons, 1989) and a ceramide kinase (Bajjalieh et al., 1989), were recovered near the void volume in low detergent concentrations (M. K. Bennett and S. Bajjalieh, unpublished observation). The fact that these markers retained enzymatic activity suggests that their native structures were preserved. The redistribution of the synaptic vesicle markers was not associated with a dramatic change in the overall protein profile across the column fractions (not shown), further indicating that the interactions leading to complex reformation are specific. In addition, the large complex recovered at low CHAPS concentration could be disrupted by subsequent treatment with Triton X-100. These observations suggest that specific interactions among 5 synaptic vesicle markers (SV2, p65, p38, $H^+$ 39 kD, and VAMP) can be promoted or stabilized by lowering the deter-

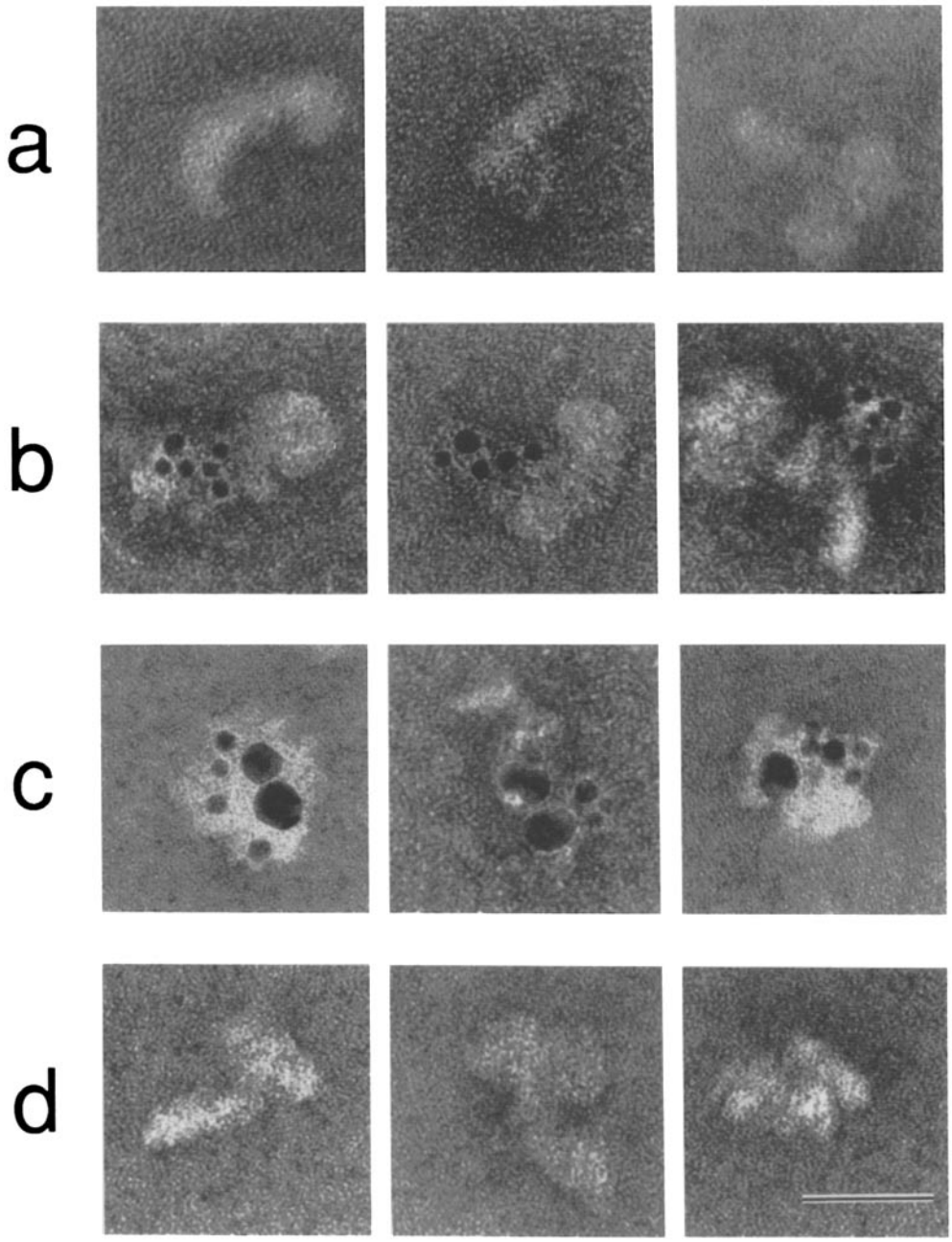


*Figure 8.* Negative stain EM of the synaptic vesicle protein complex. Synaptic vesicle fractions were solubilized in CHAPS and incubated either with no antibody (*a*), 8-nm gold–conjugated anti-p65 (*b*), 8-nm gold–conjugated anti-p65 and 20-nm gold–conjugated anti-SV2 (*c*), or 8-nm gold–conjugated anti-p65 in the presence of excess unlabeled anti-p65 (*d*). In each case, the large synaptic vesicle protein complex was isolated by gel filtration chromatography and analyzed by negative stain EM as described in Materials and Methods. Bar, 50 nm.

gent concentration, supporting the possibility that some of the interactions are hydrophobic in nature.

### *Electron Microscopy*

The morphology of the large complex of synaptic vesicle proteins was examined by transmission EM. A sample of CHAPS-solubilized vesicles was resolved on a BioGel A-5m column in the presence of 1% CHAPS. Fractions eluting near the void volume (corresponding to pool I in Fig. 6 *a*) were pooled and observed after negative staining with uranyl acetate (Fig. 8 *a*). The structures were variable in appearance, but they often consisted of two or more globular domains with either circular (~25-nm diam) or elongated (~10 × 35 nm) morphology. To determine which structures include the vesicle proteins, a CHAPS-solubilized vesicle fraction was incubated with either gold-conjugated anti-p65 (8 nm gold; Fig. 8 *b*) or gold-conjugated anti-p65 and gold-conjugated anti-SV2 (20 nm gold; Fig. 8 *c*) before gel filtration chromatography. The gold-labeled structures eluting near the void volume were similar to the structures recovered in the absence of antibody (Fig. 8 *a*). The gold-conjugated anti-p65 was always found in clusters that were associated with one of the globular domains (Fig. 8 *b*). Since each gold particle is coupled to multiple antibody molecules, the fact that an average of five gold particles were associated with each structure suggests that the complex contains multiple copies of p65. In the structures labeled with both gold-conjugated antibodies (Fig. 8 *c*), the anti-SV2 often labeled a limited domain of the structure, and the structures were less well defined, perhaps due to perturbation by antibody binding. The specificity of labeling was demonstrated by the lack of gold particles in samples that were prepared in the

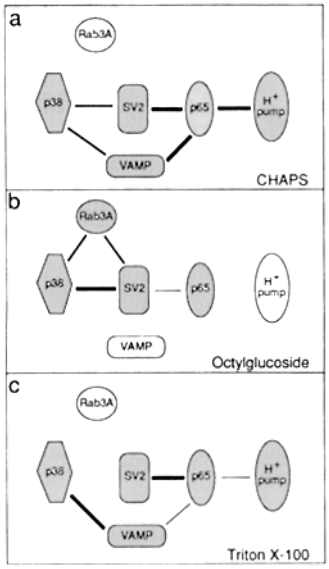


*Figure 9.* Schematic representation of interactions between synaptic vesicle proteins following detergent solubilization. Shaded objects indicate interacting proteins while nonshaded objects represent proteins that are independent of other markers. The thickness of the lines connecting the proteins indicates the relative "strength" of the interactions. For details see Discussion.

presence of excess unlabeled anti-p65 (Fig. 8 *d*). The fact that similar structures were observed in all four conditions suggests that antibody-induced cross-linking is not occurring. When anti-p65–labeled complex was isolated (as in Fig. 8 *b*), embedded in epon, and observed in thin sections, clusters of gold were again seen, but the structures were poorly preserved (not shown), indicating the absence of a high density of protein or lipid.

## Discussion

We have identified a number of interactions involving synaptic vesicle membrane proteins which are preserved following detergent solubilization. These interactions are summarized in Fig. 9. The lines connecting the interacting proteins (*shaded objects*) indicate the efficiency with which the different proteins co-isolate during fractionation. Thick lines indicate the most efficient or strongest interactions, and thin lines the least efficient. Apparent weak interactions could be the result of either unstable or low affinity binding between the components, or the participation of only subpopulations of the proteins in the interaction. The positions of the vesicle proteins in Fig. 9 were conserved among the three detergents so that the diagram may depict the relative position of each marker present in a native lipid environment. The interactions depicted are not necessarily direct, since other components of the synaptic vesicle protein complexes could be involved. The precise nature of the interactions, the amount of each marker involved, and the possible regulation of the interactions remain to be determined.

The complex of proteins preserved in CHAPS (Fig. 9 *a*) includes all of the markers tested except rab3A, but no detectable lipid. Sucrose gradient and gel filtration analysis indicated that the multicomponent complex was heterogeneous in size. In addition, some of the markers were recovered in subcomplexes (or not included in any complex) following CHAPS solubilization. These results suggest that the largest complexes may be unstable under the conditions used for isolation. The large complex of synaptic vesicle proteins appeared by negative stain electron microscopy to be composed of two or more globular domains and could be labeled with gold-conjugated antibodies against SV2 and p65 (Fig. 8). Both p65 and SV2 appeared to label limited domains of the observed structures. The other domains are likely to consist of the multimeric proton pump complex and/or oligomers of p38 (Thomas et al., 1988).

In contrast to the large multicomponent complex recovered in CHAPS, smaller complexes of synaptic vesicle membrane proteins were recovered following solubilization in octylglucoside and Triton X-100. In octylglucoside (Fig. 9 *b*) a complex of SV2, p38, and rab3A was detected by immunoprecipitation. Immunoprecipitation of octylglucoside solubilized vesicles with anti-SV2 also resulted in the specific recovery of a small amount of plasma membrane $Na^+/K^+$ ATPase. It is known that the $Na^+/K^+$ ATPase interacts with submembranous cytoskeletal components, including ankyrin and fodrin (Nelson and Hammerton, 1989). Electron microscopic evidence has indicated that fodrin may play a role in the anchoring of synaptic vesicles near the presynaptic membrane (Hirokawa et al., 1989). In addition, fodrin can regulate the fusion of chromaffin granules with the plasma membrane (Perrin et al., 1987). Perhaps the putative interaction involving SV2 and the $Na^+/K^+$ ATPase reflects an interaction of the synaptic vesicles with the fodrin cytoskeleton before exocytosis. Such an interaction between SV2 and cytoskeletal components might also explain the large size of the octylglucoside-solubilized SV2 detected on sucrose gradients. Distinct from the interactions in octylglucoside were two interactions preserved following Triton X-100 solubilization of synaptic vesicles (Fig. 9 *c*). One interaction involved predominantly SV2 and p65, with small amounts of VAMP and the proton pump, while the other involved p38 and VAMP.

In addition to the known synaptic vesicle proteins, several other proteins (as detected by silver staining) were recovered by immunoprecipitation of solubilized vesicle fractions. It will be of interest to characterize these proteins further to see if they represent novel synaptic vesicle specific proteins, or if they represent soluble, cytoskeletal, or nonvesicular membrane proteins that might specifically interact with the synaptic vesicle proteins at some point in their lifecycle.

To determine the proportion of vesicular protein present in the large complex, and to establish the number of complexes that might exist in a single vesicle, an estimate of the amount of protein/vesicle is required. The most detailed physical characterization of a synaptic vesicle preparation was carried out by Wagner et al. (1978) on vesicles isolated from the electric organ of *Narcine brasiliensis*. These ~80-nm-diam vesicles had a buoyant density of 1.05 g/ml and a protein to lipid ratio of 1:5. Based on these and other values it was estimated that each vesicle contains $\sim 6.4 \times 10^6$ daltons of protein. Extrapolation of these values to 40-nm-diam mammalian synaptic vesicles yields $\sim 1.6 \times 10^6$ daltons of protein/vesicle. However, mammalian synaptic vesicles have a higher buoyant density than electric fish vesicles (1.12 g/ml; Floor et al., 1988; Clift-O'Grady et al., 1990; Cutler and Cramer, 1990), suggesting that they have a higher protein to lipid ratio. If one assumes a protein to lipid ratio of 1:1 for mammalian synaptic vesicles, then the protein content would be $\sim 4 \times 10^6$ daltons/vesicle. Floor et al. (1990) have estimated the abundance of the vacuolar proton pump in rat brain synaptic vesicles to be ~20% of total vesicular protein. They suggest that there are six proton pump complexes

(~500 kD each) on a single vesicle. These estimates yield a value of 15 × $10^6$ daltons of protein/vesicle. Based on these data, it is reasonable to assume that a mammalian synaptic vesicle contains on the order of 4-15 × $10^6$ daltons of protein. The largest complex of synaptic vesicle proteins preserved in CHAPS had a relative sedimentation coefficient of greater than 20S, and eluted near the void volume on a gel filtration column with an exclusion limit of 5 × $10^6$ daltons. These values suggest that a large proportion of the total vesicular protein is participating in the complex, consistent with the protein composition of the multimeric complex (Fig. 4 *a*), and that the number of complexes/vesicle is three or less. The apparent size of the synaptic vesicle protein complex, as observed by negative stain EM, would occupy a significant portion of the synaptic vesicle surface, consistent with these estimates. The complex of vesicle proteins may represent a tight cluster of proteins within a restricted domain of the vesicle membrane, or a loose-knit "scaffold" spread over the vesicle surface; this remains to be established. Consistent with our results is a report that treatment of synaptic vesicles with a chemical cross-linking reagent resulted in the formation of a cross-linked complex (~5 × $10^6$ daltons) that included most of the synaptic vesicle protein (Zisapel, 1982).

The functions of synaptic vesicle proteins are likely to include intracellular targetting, recognition of and fusion with the plasma membrane, and endocytic recycling to regenerate synaptic vesicles (Südhof and Jahn, 1991). The interactions between synaptic vesicle proteins we observe have a number of implications with regard to these functions. Sorting and targetting of synaptic vesicle proteins is likely to occur both at the *trans*-Golgi network during biogenesis and at the plasma membrane and/or early endosome during recycling. Little is known about the molecular signals which govern the targetting of synaptic vesicle proteins. There is no indication of a consensus targetting signal from the primary sequences of synaptic vesicle proteins cloned to date. The putative targetting signal may be represented by a common secondary or tertiary structure, as with the signal for mannose phosphorylation of lysosomal hydrolases (Baranski et al., 1990). In this case, no common primary sequence would be detectable. Recent experiments in which p38 was expressed in non-neuronal cells indicate that this protein is targetted to endocytic vesicles (Leube et al., 1989; Johnson et al., 1989; Linstedt and Kelly, 1991). This result suggests that p38 contains some targetting information. One possibility that is raised by our results is that targetting, both during synaptic vesicle biogenesis and during endocytic recycling, is mediated by a single vesicle protein with primary targetting information. Other vesicle proteins could be targetted by virtue of interactions with this protein. p38 is a good candidate for such a targetting protein, given its localization following expression in non-neuronal cells, and its apparent interactions with other synaptic vesicle proteins (Fig. 9).

Another important aspect of synaptic vesicle protein function is the process of docking and fusion. A population of synaptic vesicles is closely associated with the presynaptic plasma membrane. These vesicles fuse very rapidly after $Ca^{2+}$ influx, suggesting that the cellular machinery required for membrane fusion is already assembled. The molecular mechanisms involved in cellular membrane fusion events are not well defined. Studies of secretory mutants in yeast and the use of cell-free assays for transport vesicle fusion have led to the identification of a number of factors that are required for membrane fusion (Wattenberg and Rothman, 1986; Malhotra et al., 1988; Eakle et al., 1988; Weidman et al., 1989; Clary et al., 1990; Goda and Pfeffer, 1991). Several of these factors appear to be soluble or peripheral membrane components that assemble into a pre-fusion complex. It is possible that some of the interactions observed between the different synaptic vesicle proteins represent integral membrane constituents of such a pre-fusion complex. It will be of interest to determine if any of the other proteins in the synaptic vesicle protein complexes are related to the proteins proposed to be part of the fusion machinery in other systems.

The large complex of vesicle proteins recovered following CHAPS solubilization could play an important role in the recycling of synaptic vesicle membrane proteins. The mechanism by which synaptic vesicles are regenerated following exocytosis is not well established. One model suggests that the vesicle membrane becomes fully incorporated into the plasma membrane and that recycling requires an endocytic process involving coated pits, coated vesicles, and an early endosome compartment (Heuser, 1989). Another model proposes that exocytosis occurs by the formation of a fusion pore, allowing vesicle contents to be released, and that recycling occurs by simple closure of the fusion pore and pinching off of the vesicle (Meldolesi and Ceccarelli, 1981; Torri-Tarelli et al., 1987). If recycling occurs by way of a coated vesicle pathway, the synaptic vesicle protein complex would simplify the selection of endocytic cargo, thereby increasing the efficiency of the process. The observations that rab3A recycles by means of a cytosolic intermediate (Fischer von Mollard et al., 1991), and that rab3A is excluded from the large complex of vesicle proteins, is consistent with a role of the large complex in endocytic recycling of integral synaptic vesicle membrane proteins. If exocytosis and recycling normally occur by means of opening and closing of a fusion pore, the complex of vesicle proteins could play a role in maintaining vesicle shape, preventing collapse into the plasma membrane.

The very small size of mammalian synaptic vesicles is intriguing. It has been proposed that the co-expression of multiple vesicle proteins may be required to obtain this small size, since the vesicles that accumulate following expression of p38 in non-neuronal cells are much larger than authentic synaptic vesicles (Clift-O'Grady et al., 1990). The proposed interactions among the synaptic vesicle proteins may be responsible for maintaining the size and shape of synaptic vesicles, which in turn may be functionally important for the processes of membrane fusion and recycling.

The multiple interactions between synaptic vesicle membrane proteins we describe here are likely to be relevant to several aspects of synaptic vesicle function. It will be important to establish at which stages of the synaptic vesicle lifecycle the various interactions occur. Equally important is the possibility that these interactions could be the target of regulatory mechanisms that modulate synaptic transmission. We are now in a position to further evaluate the nature of the interactions and to study their possible physiological regulation.

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