Identification of a Transmembrane Glycoprotein Specific for Secretory Vesicles of Neural and Endocrine Cells

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ABSTRACT Several types of cells store proteins in secretory vesicles from which they are released by an appropriate stimulus. It might be expected that the secretory vesicles in different cell types use similar molecular machinery. Here we describe a transmembrane glycoprotein (M_r ~100,000) that is present in secretory vesicles in all neurons and endocrine cells studied, in species from elasmobranch fish to mammals, and in neural and endocrine cell lines. It was detected by cross-reactivity with monoclonal antibodies raised to highly purified cholinergic synaptic vesicles from the electric organ of fish. By immunoprecipitation of intact synaptic vesicles and electron microscopic immunoperoxidase labeling, we have shown that the antigenic determinant is on the cytoplasmic face of the synaptic vesicles. However, the electrophoretic mobility of the antigen synthesized in the presence of tunicamycin is reduced to M_r ~62,000, which suggests that the antigen is glycosylated and must therefore span the vesicle membrane.

It now appears likely that protein secretion by cells can take two forms (14, 16, 38). Constitutive secretion, in which the rate of protein secretion is unregulated and quite closely follows the rate of protein synthesis, occurs in almost all cell types, including liver cells, lymphocytes, and yeast cells. In contrast, the rate of secretion is highly regulated in endocrine, exocrine, and neuronal cells which respond to external signals by releasing a large amount of protein in a short period of time. Cells with regulated secretion store their secretory proteins in storage granules, whose probability of fusing with the plasma membrane is dramatically enhanced by the presence of a chemical or electrical signal. Some cells appear to have both constitutive and regulated pathways (14, 16). Cells that have the regulated secretory pathway must express gene products unique to this pathway, and proteins necessary for storage of secretory proteins, for calcium-dependent exocytosis, and for sorting of secretory proteins between the constitutive and regulated pathway, for example. Because of differences among the exocrine, endocrine, and neuronal cells, one might also expect to find proteins specific for the secretory pathway in only one of the cell types.

One potential way of identifying proteins involved in the regulated pathway is to generate antibodies specific for the storage vesicles characteristic of that pathway. This approach has been successful with other subcellular organelles. For instance, both polyclonal and monoclonal antibodies now exist that allow the relatively unambiguous recognition of clathrin-coated membranes (27), rough endoplasmic reticulum (28, 39), and the Golgi apparatus (6, 26, 28, 37, 39). The possibility of using a similar approach to identify universal components of secretory vesicles was suggested by the observation that a polyclonal antiserum to synaptic vesicles purified from the electric organ of a marine electric ray recognized not only nerve terminals in many areas of the mammalian brain, but also endocrine cells (18). We have therefore prepared a library of monoclonal antibodies that recognize unique antigens in synaptic vesicles. By screening for an antigen shared by secretory vesicles in other cells with the regulated pathway we found that one monoclonal antibody (anti-SV2) recognizes a transmembrane glycoprotein that appears to be common to all synaptic vesicles and endocrine secretory vesicles. The glycoprotein can be detected in nerve terminals of species ranging from rat and frog to cartilagenous fish. Although exocrine cells have regulated secretion, they lack the antigen.

The antibody and the protein it recognizes may provide a marker for the appearance and disappearance of the regulated pathway in neurons and endocrine cells. Because the antigenic site is on the cytoplasmic surface, the antibody is also useful for purification by immunoadsorption of membranous organelles that contain the glycoprotein. For example, it has already permitted the isolation of a subclass of bovine brain coated vesicles that contains synaptic vesicle proteins (33).

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MATERIALS AND METHODS

Materials: [^{35}S]methionine was obtained from Amersham Corp. (Arlington Heights, IL). Rabbit anti-mouse Immunobeads and protein assay reagents were purchased from Bio-Rad Laboratories (Richmond, CA). Nitrocellulose filters were obtained from Sartorius Filters, Inc. (Hayward, California), polyethylene glycol 4000 from Merck A. G. (Darmstadt, FRG), fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated sheep anti-rabbit IgG from Cappel Laboratories (West Chester, PA), horseradish peroxidase-conjugated goat anti-mouse IgG and Tissue-Tek from Miles Laboratories Inc. (Elkhart, IN), and ultrapure urea from Schwarz/Mann (Spring Valley, NY). 10 nm gold-goat anti-mouse IgG was obtained from Jansen Pharmaceutica, Structure Probe Inc. (West Chester, PA), and Lowicryl K4M was purchased from Polysciences, Inc. (Warrington, PA). All remaining chemicals, including molecular weight standards, were obtained from Sigma Chemical Co. (St. Louis, MO).

Nerve growth factor (NGF)[1] was generously donated by Dr. David Shelton, University of California, San Francisco (UCSF).

Preparation of Monoclonal Antibodies: Synaptic vesicles were purified from the elasmobranch *Discopyge ommata* as described for *Narcine brasiliensis* by Carlson et al. (10). Spleen cells from BALB/c mice immunized with synaptic vesicles were fused with SP2/O myeloma cells by use of polyethylene glycol 4000 according to the modifications by Fazekas de St. Groth and Sheiddegger (12) of the procedure of Kohler and Milstein (22). Supernatants from the hybridomas were screened for the presence of synaptic vesicle specific antibodies by use of a solid-phase radioimmunoassay that compares binding of antibodies to elasmobranch synaptic vesicles and to a vesicle-free side fraction from the controlled pore glass column (8).

Immunocytochemistry: Tissues from animals perfused with 4% formaldehyde were infiltrated with phosphate-buffered saline (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) (PBS) containing 10% sucrose, mounted in Tissue-Tek, quick-frozen with dichlorodifluoromethane, and sectioned on a cryostat. Cells in culture were plated on poly-*d*-lysine-coated glass coverslips, and fixed with 3.7% formalin in PBS for immunofluorescence. Monolayers of fixed cells or 10–12 μm cryostat sections were preincubated in 1% bovine serum albumin in PBS (BSA-PBS) for 5 min, then incubated with undiluted hybridoma supernatant for 1 h. After three brief rinses with 1% BSA-PBS, fluorescein-conjugated goat anti-mouse IgG in 1% BSA-PBS was added for 1 h. Sections and coverslips were washed with 1% BSA-PBS and PBS, mounted in 90% glycerol, 10% PBS, 2.5% 1,4 diazobicyclo[2,2,2]octane (20) and viewed with fluorescein filters. When horseradish peroxidase–conjugated IgG was used as a second antibody on cryostat sections, 0.1% Triton X-100 was used in the 1% BSA-PBS buffer throughout the incubations and washes. After they were rinsed with PBS, the sections were reacted with 0.5 mg/ml diaminobenzidine hydrochloride and 0.01% hydrogen peroxide in 0.05 M Tris, pH 7.6, until reaction product was visible. The sections were then dehydrated in ethanol, cleared in xylene, and mounted in Permount (Fisher Scientific Co., Fair Lawn, NJ).

AtT-20 cells were stained for adrenocorticotropin (ACTH) with rabbit antiserum generated as described by Moore et al. (32) and affinity purified according to the method of Mains and Eipper (29). The cells were fixed with 3.7% formalin, pretreated with 0.25% Triton X-100 in PBS-BSA, and incubated with antiserum to ACTH, followed by rhodamine-conjugated sheep anti-rabbit IgG. All rinses and antibody incubations were done in 0.25% Triton X-100 in PBS-BSA.

Immunoelectronmicroscopy of electric organ nerve terminals was done as described (5).

Immunocytochemistry of Lowicryl-embedded tissue was done by a modification of the procedure of Altman et al. (1) and is described in detail by Valentino et al. (Valentino, K. L., D. A. Crumvine, and L. F. Reichardt, manuscript submitted for publication). In short, rat brains were fixed by perfusion with 4% paraformaldehyde, 0.1% glutaraldehyde, and 2 mM $CaCl_2$ in 0.1 M cacodylate buffer. Brains were removed and fixed overnight in the same fixative but without glutaraldehyde. Pieces of cerebellum were washed in 0.1 M cacodylate buffer and stained *en bloc* with 2% aqueous uranyl acetate at 4°C for 1–2 h. Tissue was dehydrated in graded series of ethanol and infiltrated with Lowicryl K4M as described (Valentino, K. L., D. A. Crumvine, and L. F. Reichardt, manuscript submitted for publication). Thin sections were collected on Formvar-coated nickel grids, washed with 5% normal goat serum in PBS, and incubated with undiluted hybridoma supernatant for 20 min. After a brief rinse with 5% normal goat serum-PBS, the sections were floated on a drop of undiluted goat anti-mouse IgG adsorbed to 10 nm colloidal gold for 5 min. Control sections were incubated with culture medium and goat anti-mouse IgG-colloidal gold. Grids were stained with 2% OsO_4 and with 2% uranyl acetate.

Random fields from both the glomerular and molecular layers of the cerebellum were photographed at 15,200 X and printed at a further magnification of 2.5. 12 micrographs were chosen in which synapses could be clearly recognized by the presence of pre- and postsynaptic densities. Presynaptic terminals with intact plasma membranes (a total of 55 terminals) were outlined with ink. On each micrograph, the total number of gold particles and the number of gold particles localized over the outlined synapses were counted. The micrographs were then Xeroxed and weighed before and after the outlined synapses were cut out. The total number of gold particles in all 12 micrographs (3,293 particles) was divided by the total weight of the micrographs. This figure represents the density of the gold particles if they are randomly distributed but does not represent background binding, since synaptic binding is included in this number. The number of gold particles localized over the outlined synaptic terminals was divided by the total weight of the synapses. An increase in the density of gold particles associated with synaptic terminals over average density represents a specific association of gold particles with presynaptic structures. Control sections incubated with culture medium and goat anti-mouse IgG-colloidal gold had <10 gold particles per micrograph.

Immunoblots: Samples were analyzed by SDS PAGE (8% acrylamide) in 8 M urea. Proteins were transferred from the gels to nitrocellulose paper as described by Burnette (7) for 6–24 h. The nitrocellulose filters were incubated with hybridoma supernatant diluted 1:50 in 5% BSA-Tris saline buffer (150 mM NaCl, 100 mM Tris), rinsed, and then incubated with ^{125}I-labeled goat anti-mouse IgG (~10^6 cpm per 8 × 10 cm filter) in 5% BSA-Tris saline.

Preparation of Tissues for Immunoblots: Synaptosomes were isolated from bovine brain by a modification of published procedures (13). The tissue was homogenized in a buffer of 0.32 M sucrose, 10 mM HEPES, and 10 mM EGTA, pH 7.4 (2 g brain/10 ml buffer). The pellet obtained by centrifugation of the homogenate at 20,000 *g* for 20 min was washed by centrifugation and resuspension in homogenization buffer. 15-ml samples of the resuspended material were centrifuged at 80,000 *g* for 16 h in a discontinuous Ficoll gradient consisting of 8-ml layers of 2, 8, and 16% Ficoll in homogenization buffer. The synaptosome band was collected from the interface between 8 and 16% Ficoll. Chromaffin granule membranes prepared by the method of Trifaro and Dworkind (40) were a gift of Dr. Erik Schweitzer; membrane fractions from rabbit and chicken brain were generously donated by Dr. John Bixby, UCSF.

Cell Culture: AtT-20/D16V cells were grown in 10% horse serum in Dulbecco's minimal essential medium containing 4.5 g/liter glucose (Dulbecco's minimum essential medium-H21) at 15% CO_2. The pheochromocytoma cell line, PC12, was obtained from UCSF tissue culture facility and grown in 10% fetal calf serum and 5% horse serum in Dulbecco's minimum essential medium-H21 without NGF, except for immunofluorescence studies where 2.5S NGF was added at a concentration of 100 ng/ml; HIT cells were grown in 15% heat-inactivated horse serum and 2.5% fetal calf serum in Dulbecco's minimum essential medium-H16 (1 g/liter glucose); and GH3 cells were grown in 10% fetal calf serum in Dulbecco's minimum essential medium-H21 at 10% CO_2. Superior cervical ganglion neurons, dissociated from 1–2-d-old rats, were generously donated by Arthur Lander (UCSF). The neurons were plated in medium containing NGF on poly-*d*-lysine–coated glass coverslips which had been pretreated with bovine corneal epithelium conditioned medium as previously described (24) and fixed 24 h after plating for immunofluorescence.

Metabolic Labeling with [^{35}S]Methionine: PC12 cells were grown to confluence without NGF in 15 cm tissue culture plates. Cells were labeled at 37°C with 1 mCi [^{35}S]methionine/plate in medium containing one-twentieth the normal amount of methionine. For long term labeling, cells were incubated for 5 h in [^{35}S]methionine and extracted immediately, or chased for 1 h in normal medium and then extracted. Cells treated with tunicamycin were incubated with 5 μg/ml tunicamycin in normal medium for 2 h before labeling was done. This concentration of tunicamycin does not inhibit protein synthesis in AtT-20 cells (A. Katzen and H.-P. Moore, unpublished observations). For short term labeling (<1 h), cells were first incubated for 20 min in medium with one-twentieth normal methionine. Cells were extracted with 2 ml/15 cm plate of ice-cold lysis buffer (1% Nonidet P40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris-HCl, pH 7.4 [35]) containing 0.3 mg/ml phenylmethylsulfonyl fluoride and 0.3 mg/ml iodoacetimide. The cell extracts were clarified by centrifugation at 12,000 *g* for 5 min, and the supernatants were kept for analysis.

Preparation of Monoclonal Antibody Immunobeads and Immunoprecipitation of SV2 Antigen: A 1-ml slurry of 10 mg/ml rabbit antimouse Immunobeads was mixed with 2 ml of monoclonal supernatant overnight at 4°C. After incubation with the monoclonal antibody, the beads were washed three times by centrifugation and resuspension in the original volume (10 mg/ml) of immunoprecipitation buffer (1% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, 0.4 M NaCl, pH 7.4). Immunoprecipitations were done by a modification of the procedure of Carlson

[1] *Abbreviations used in this paper*: mAb, monoclonal antibody; NGF, nerve growth factor.

and Kelly (9). Samples to be precipitated were dissolved in 1% SDS, then diluted 10-fold with 0.1% BSA in immunoprecipitation buffer, and mixed with 50 μl of immunobeads/20 μg protein overnight at 4°C. Control beads were prepared with a monoclonal antibody to a glycoprotein of elasmobranch synaptic vesicles (SV1) that does not crossreact with the mammalian nervous system.

Immunoprecipitation of Synaptic Vesicles: Immunoprecipitation of whole and sonicated vesicles labeled by iodination with [^{125}I]diazotized iodosulfanilic acid was done as described by Carlson and Kelly (9) with the following modifications. Aliquots of labeled vesicles were incubated overnight with two different concentrations of monoclonal supernatant (0.5 or 1.0 μl) or culture medium without antibodies at 4°C. 50 μl of a 10 mg/ml slurry of rabbit antimouse Immunobeads was then added to each sample and incubated with rotation for 3 h at 4°C. The labeled beads were spun for 10 min at 12,000 *g* through a pad of 12.5% Ficoll in 0.4 M NaCl, 10 mM HEPES, 1% BSA, pH 7.0, and washed three times with buffer. The pelleted beads were counted and the amount of radiolabeled material associated with the beads was expressed as a percentage of the total tricholoracetic acid–precipitable radioactivity present in the starting material. There was no significant difference in the percent of trichloroacetic acid–precipitable counts bound to Immunobeads prepared with two different concentrations of monoclonal supernatant.

Other Techniques: Proteins were determined by the method of Bradford (4) by use of the Bio-Rad protein assay reagent with BSA as a standard. Approximate M_r in SDS PAGE was determined by preparation of a calibration curve based on the mobilities of myosin (205,000), beta-galactosidase (116,000), phosphorylase B (97,400), bovine albumin (66,000), egg albumin (45,000), and carbonic anhydrase (29,000).

RESULTS

Monoclonal Antibodies Specific for Electric Organ Synaptic Vesicles

We have generated monoclonal antibodies to unique components of cholinergic synaptic vesicles. Mice were immunized with highly purified vesicles from the electric organ or the elasmobranch *Discopyge ommata* and hybridoma cell lines were obtained by conventional techniques. Antibodies secreted by these cell lines were tested for specific binding to purified electric organ synaptic vesicles using a solid-phase radioimmunoassay. In a panel of six monoclonal antibodies specific for synaptic vesicles by solid-phase radioimmunoassay, antibodies to one antigen, SV2, also showed wide cross-reactivity with the mammalian nervous system. Antibodies to SV2 were further screened to show that the antibodies recognized on antigenic determinant that co-purified with elasmobranch synaptic vesicle contents during permeation chromatography on controlled pore glass columns (Fig. 1). To establish the location of the SV2 antigen in synaptic vesicles purified from the electric organ, we tested hybridoma supernatant to this antigen for its ability to precipitate intact or sonicated vesicles. Antibodies to the SV2 antigen were able to precipitate both intact and sonicated vesicles, whereas antibodies to a proteoglycan-like material (SV1) that is on the inside of synaptic vesicles (9) were able to precipitate vesicle membranes only when the vesicles had been sonicated previously (Fig. 2). We saw consistent results with electron microscopy (Fig. 3) using an immunoperoxidase technique to visualize antibody binding to nerve terminals in a preparation of gently homogenized electric organ (5). When nerve terminals were incubated with antibodies to the SV2 site, little or no reaction product was found associated with intact nerve terminals. Occasionally, however, synaptic vesicles were heavily labeled with reaction product in nerve terminals that were apparently lysed during preparation (Fig. 3*b*). In contrast, antibodies to the SV1 site (Fig. 3*a*) bound to the outside of nerve terminals as we have previously described (5). This antigen, which is on the inside of synaptic vesicles (9), is accessible on the outside of nerve terminals, presumably as a result of exocytosis of vesicle membrane. Unlike the SV2 antigen, the SV1 antigen was not detectable in damaged nerve terminals, presumably because the vesicle membrane remains intact. Thus, the SV2 antigenic site is on the cytoplasmic face of synaptic vesicle membranes whereas the SV1 site is on the luminal face of the vesicle membrane.

The SV2 Antigenic Site Is a Nerve Terminal Marker in Mammalian and Amphibian Nervous Systems

Monoclonal antibodies to electric organ synaptic vesicle

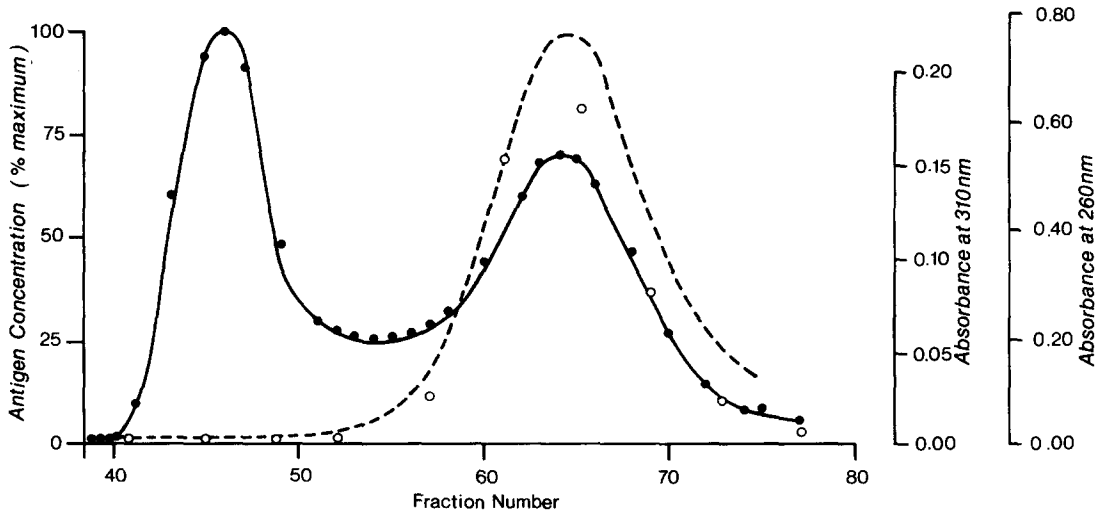


FIGURE 1 Co-purification of monoclonal antibody binding with synaptic vesicles during permeation chromatography. A sucrose gradient enriched fraction (10) was further fractionated on controlled pore glass 3000. Elution of membranes was monitored by light scattering at 310 nm (●——●) and ATP content of synaptic vesicles was monitored by absorbance at 260 nm (- - -) corrected for light scattering (9). The antigenicity of every other column fraction was measured by solid-phase radioimmunoassay on microtiter plates (8). Each open circle represents the average of two adjacent data points. The binding of anti-SV2 (○) to vesicle membranes coelutes quite closely with the peak of vesicle contents. The maximum binding of anti-SV2 was 4,000 cpm/μl.

antigens were screened for cross-reactivity with neural tissue of other species by indirect immunofluorescence on cryostat sections of formaldehyde-fixed rat spinal cord. Only the monoclonal antibody to the SV2 antigen showed detectable binding. The pattern of binding seen with this antibody suggests that it is not restricted to any one class of nerve terminals but rather is a universal marker for vertebrate nerve terminals. Small, punctate fluorescence was seen in all regions of the rat and frog spinal cord. Label was particularly heavy in the substantia gelatinosa of the dorsal horn, and, in the ventral horn of the spinal cord, the cell bodies and dendrites of the motor neurons were outlined by fluorescent spots (Fig. 4, *a* and *b*). Little specific fluorescence was seen in the neuronal cytoplasm. The antigen was not restricted to neurons that innervate the peripheral nervous system because the monoclonal antibody to SV2 bound to both the glomerular and molecular layers of the rat cerebellum and all the synaptic layers of the hippocampus (Fig. 4, *c* and *d*). The SV2 antigen was also found in amphibian central nervous system, as monoclonal antibody to SV2 bound to the synaptic regions of the bullfrog retina, the inner and outer plexiform layers (Fig. 4, *e* and *f*), and to synapses in the frog spinal cord (data not shown).

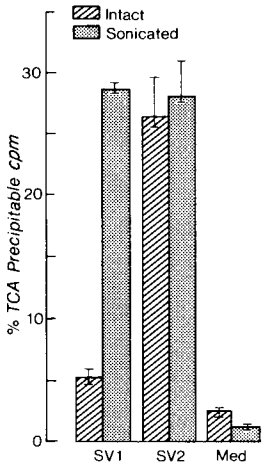


FIGURE 2 Immunoprecipitation of intact and sonicated vesicles with monoclonal antibodies bound to Immunobeads. ^{125}I-labeled intact or sonicated synaptic vesicles were incubated with monoclonal antibodies bound to Sepharose beads (SV1 or SV2). Control beads (*med*) were preincubated with culture medium without antibodies. Each bar is the average of duplicate samples in one experiment. The percentage of total trichloroacetic acid-precipitable radioactivity (78,526 cpm) immunoprecipitated by each antibody is shown for intact or sonicated vesicles.

To obtain further evidence for the association of SV2 with synaptic vesicles in the mammalian nervous system, we examined the binding of antibodies to SV2 to thin sections of Lowicryl-embedded rat cerebellum and visualized the binding sites with goat anti-mouse IgG adsorbed to colloidal gold. Gold particles were concentrated over presynaptic elements, which could be easily identified by the darkly stained pre- and postsynaptic densities (Fig. 5, *a–c*), and also over varicosities (Fig. 5*a*). We found few gold particles over mitochondria within nerve terminals (Fig. 5, *b* and *c*), which suggests that the antigen is associated predominantly with synaptic vesicles. To obtain some quantitative measure of the synaptic specificity of antibody binding in the cerebellum, we compared the density of gold particles over synaptic terminals to the density of the total number of gold particles in a given field, as described in Materials and Methods. We chose this method of comparison because the preservation of ultrastructural detail was variable throughout the section. Consequently, the binding sites of all the gold particles could not be assigned unambiguously to synaptic or nonsynaptic structures. The density of gold particles located over presynaptic terminals was 4.2 times greater than the average density of gold particles. Furthermore, control sections incubated with culture medium alone had <4% of the total number of gold particles seen with antibodies to SV2.

Cross-reaction with Other Secretory Cells

Because cells with the regulated secretory pathway are similar in many ways, we asked if the SV2 antigen was also present in secretory vesicles of non-neural secretory cells. The antibody showed specific binding to cells in several types of endocrine tissues in the rat, including the adrenal, pituitary, and pancreas. In the adrenal gland, the antibody bound to the endocrine cells of the medulla but not to the steroid-

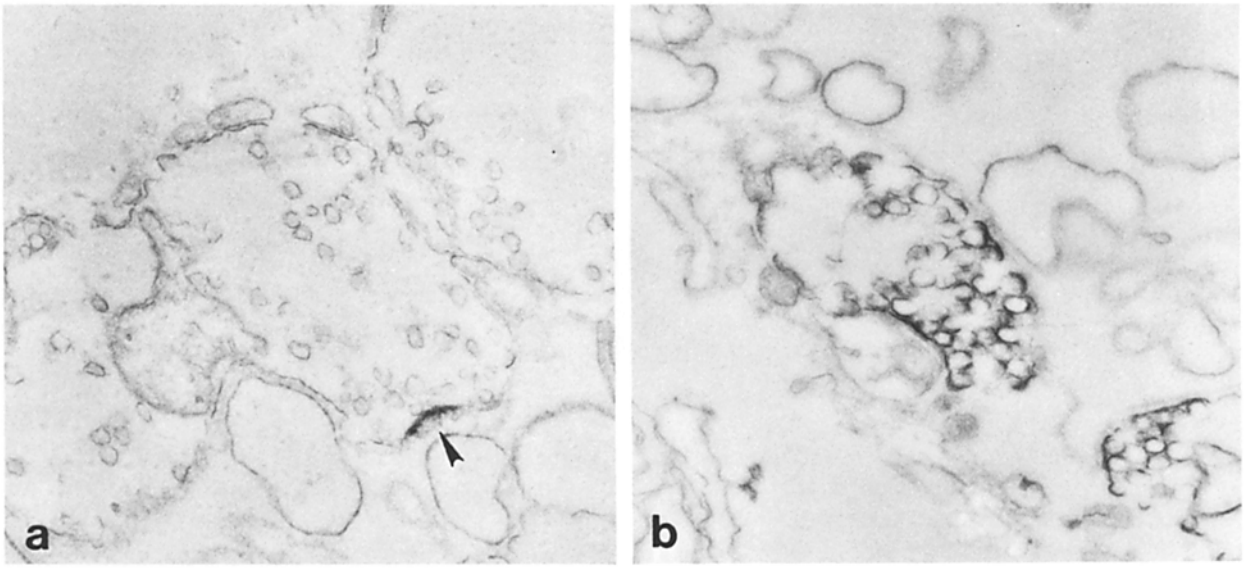


FIGURE 3 Localization of antibody binding sites on intact and disrupted nerve terminals with immunoperoxidase techniques. After incubation of electric organ nerve terminals with monoclonal antibodies to SV1 (*a*), peroxidase reaction product (arrowhead) was associated with the outside of the presynaptic membrane. In contrast, after incubation with antibody to SV2 (*b*), the reaction product appeared to be localized to the outside of synaptic vesicles in nerve terminals that were apparently lysed during preparation of the tissue. × 31,920.

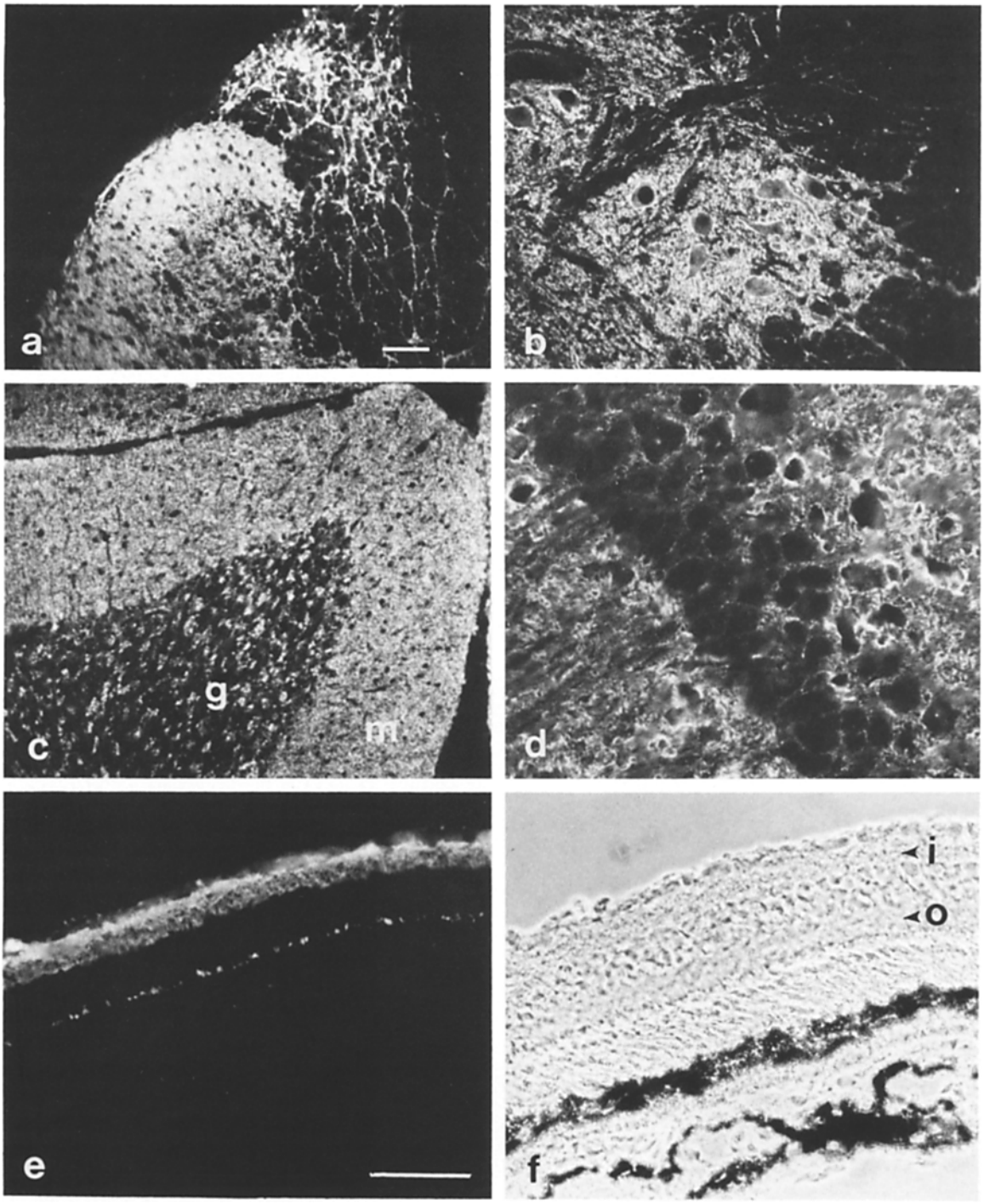


FIGURE 4 Immunofluorescent staining of mammalian and amphibian nerve terminals with monoclonal antibody to SV2. (*a*) Dorsal horn of rat spinal cord. (*b*) Ventral horn of rat spinal cord. (*c*) Rat cerebellum; *g*, granular layer; *m*, molecular layer. (*d*) CA1 region of the rat hippocampus. (*e*) Bullfrog retina. (*f*) Corresponding phase picture of *e*; *i*, inner plexiform layer; *o*, outer plexiform layer. (*a–c*) Bar, 50 μm. × 140. (*d–f*) Bar, 50 μm. × 300.

producing cells of the cortex, presumably because they lack secretory vesicles (Fig. 6*c*). It also bound strongly to cells of the intermediate lobe of the pituitary and less strongly, but detectably, to the cells of the anterior lobe (Fig. 6, *a* and *b*).

The pancreas is composed of two types of secretory cells. The acinar cells, classified as exocrine, surround small groups of islet cells which are considered endocrine. In sections of pancreas, only the islet cells (clearly distinguishable in phase microscopy by the absence of the phase dense granules present in the acinar cells [Fig. 6*f*]) showed fluorescent staining after incubation with the monoclonal antibody to SV2, whereas the acinar cells had no detectable fluorescence (Fig. 6, *d* and *e*). It would seem therefore that the SV2 antigen is not a universal marker for cells with regulated secretion but is specific for neurons and endocrine cells. Other secretory tissues that did not contain detectable amounts of the SV2 antigen were rat submaxillary salivary gland, thyroid follicular cells, liver, and lymph node (data not shown).

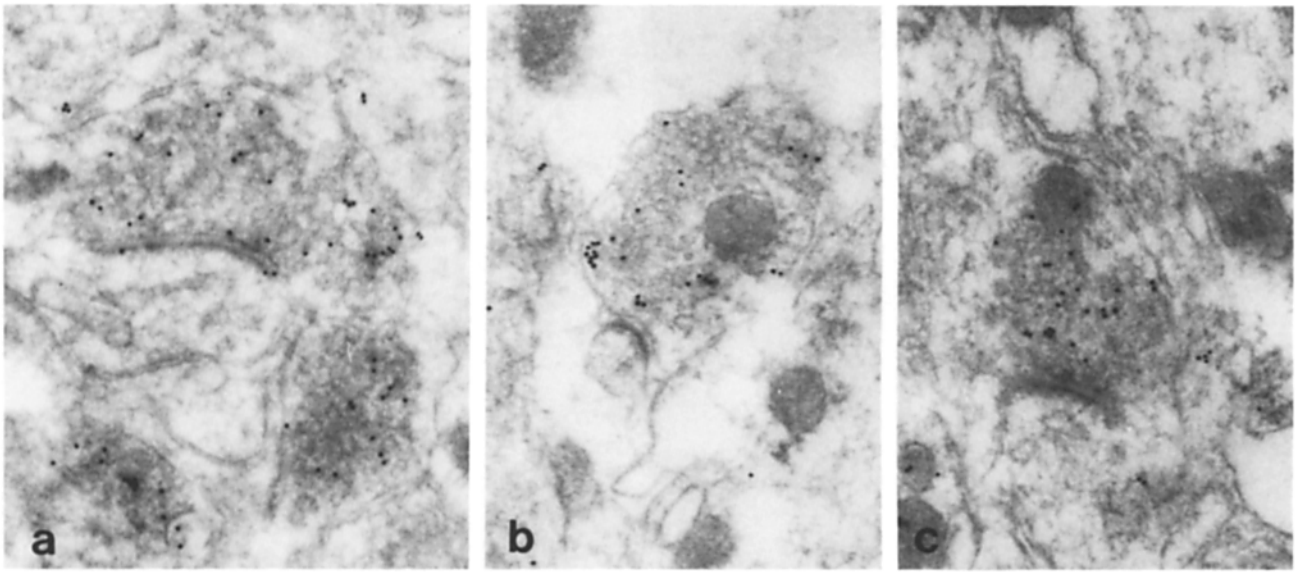


FIGURE 5 Localization of binding sites of antibody to the SV2 glycoprotein in rat cerebellum with colloidal gold. Gold particles are concentrated over presynaptic terminals (a–c) but can also be seen over structures resembling varicosities (a). × 50,160.

Expression of the SV Antigen in Cultured Cells

To study the biogenesis and function of the SV2 antigen it would be an obvious advantage if the antigen were expressed in neural and endocrine cells in culture. Several cell types were tested and found to have the SV2 antigen. Neurons dissociated from superior cervical ganglia of neonatal rats were plated into NGF and fixed 24 h later during the period of maximal neurite outgrowth. Specific immunofluorescence was seen in some, but not all, of the growth cones (Fig. 7 *d*). Furthermore, a perinuclear staining could be seen in the neuronal cells which may represent an accumulation of antigen in the Golgi region (Fig. 7 *b*).

The SV2 antigen was also present in established cell lines. The pheochromocytoma cell line, PC12, was grown for a week in the presence of NGF. After incubation with monoclonal antibodies (mAb) to SV2, there was strong fluorescence of the varicosities in these cultures and in the perinuclear region of the cell cytoplasm (Fig. 8 *a*). Three endocrine cell lines also stained with mAb to SV2. AtT-20 cells, derived from mouse anterior pituitary, synthesize, store, and secrete the hormone ACTH (15, 34). These cells bound antibody to SV2 specifically at the tips of the short processes, where secretory vesicles are known to accumulate (21) and in the perinuclear cytoplasm, perhaps associated with the Golgi apparatus (Fig. 8 *c*). This pattern of staining was similar to the pattern seen when AtT-20 cells were incubated with an affinity-purified antiserum to ACTH (Fig. 8 *d*). GH3 cells, also derived from the anterior pituitary, which secrete growth hormone and prolactin, and HIT cells, an insulin-secreting cell line, both contained the antigen by immunofluorescence techniques (Fig. 8, *b* and *e*).

Identification of SV2 as a Glycoprotein

In the electric fish synaptic vesicles, the SV2 antigen had the broad electrophoretic mobility often associated with a glycosylated protein (Fig. 9, lane *c*). To determine the biochemical characteristics of the mammalian SV2 antigen, we compared the mobility of the SV2 antigen from a variety of tissues in SDS PAGE by Western blotting techniques. Fig. 9 shows that in all samples, the SV2 antigen migrates as a similarly broad band. The predominant species in the mammalian and avian brains had M_r's of ~75,000 to 85,000 (Fig. 9, lanes *d–g*). SV2 antigen from endocrine cells appeared to have a higher molecular weight range of ~105,000–110,000, regardless of the species (Fig. 9, lanes *h–j*). The M_r of the SV2 antigen in electric fish brain (Fig. 9, lane *a*) and electromotor nucleus (Fig. 9, lane *b*) was slightly lower than the M_r of SV2 antigen in purified synaptic vesicles (Fig. 9, lane *c*). To determine if the wide range of apparent molecular weight could arise because the SV antigen is highly glycosylated, we grew PC12 cells in the presence of tunicamycin, which prevents the addition of N-linked sugars (19), and [^{35}S]methionine. After tunicamycin treatment, the antigen immunoprecipitated by mAb to SV2 appeared as a single band at ~62,000 M_r (Fig. 10, lower arrowhead, lane *c*), in contrast to the antigen from untreated cells, which ran at ~110,000 (Fig. 10, arrow, lane *b*). Both were absent in the control (lane *d*).

The identity of the 62,000 M_r protein as a nonglycosylated or precursor form of the SV2 antigen was supported by comparison of the mobility of labeled material extracted from cells immediately after a 30–45 min pulse of [^{35}S]methionine to material from cells that were labeled for 5 h and then chased for an hour in medium without [^{35}S]methionine. Fig. 10 shows that after short term labeling the SV2 antigen appeared as a single band at lower molecular weight (~64,000, lane *a*), whereas the higher molecular weight form appeared (lane *b*) after much longer labeling.

DISCUSSION

The similarities between the calcium-regulated exocytotic events in neuronal, endocrine, and exocrine cells suggests that some of the molecular machinery involved in regulated release might be shared. If the shared molecules are highly conserved then there should be common antigenic determinants in all cells that have the regulated pathway. In searching for such a determinant, we have identified a transmembrane glycoprotein present in highly purified synaptic vesicles from elasmo-

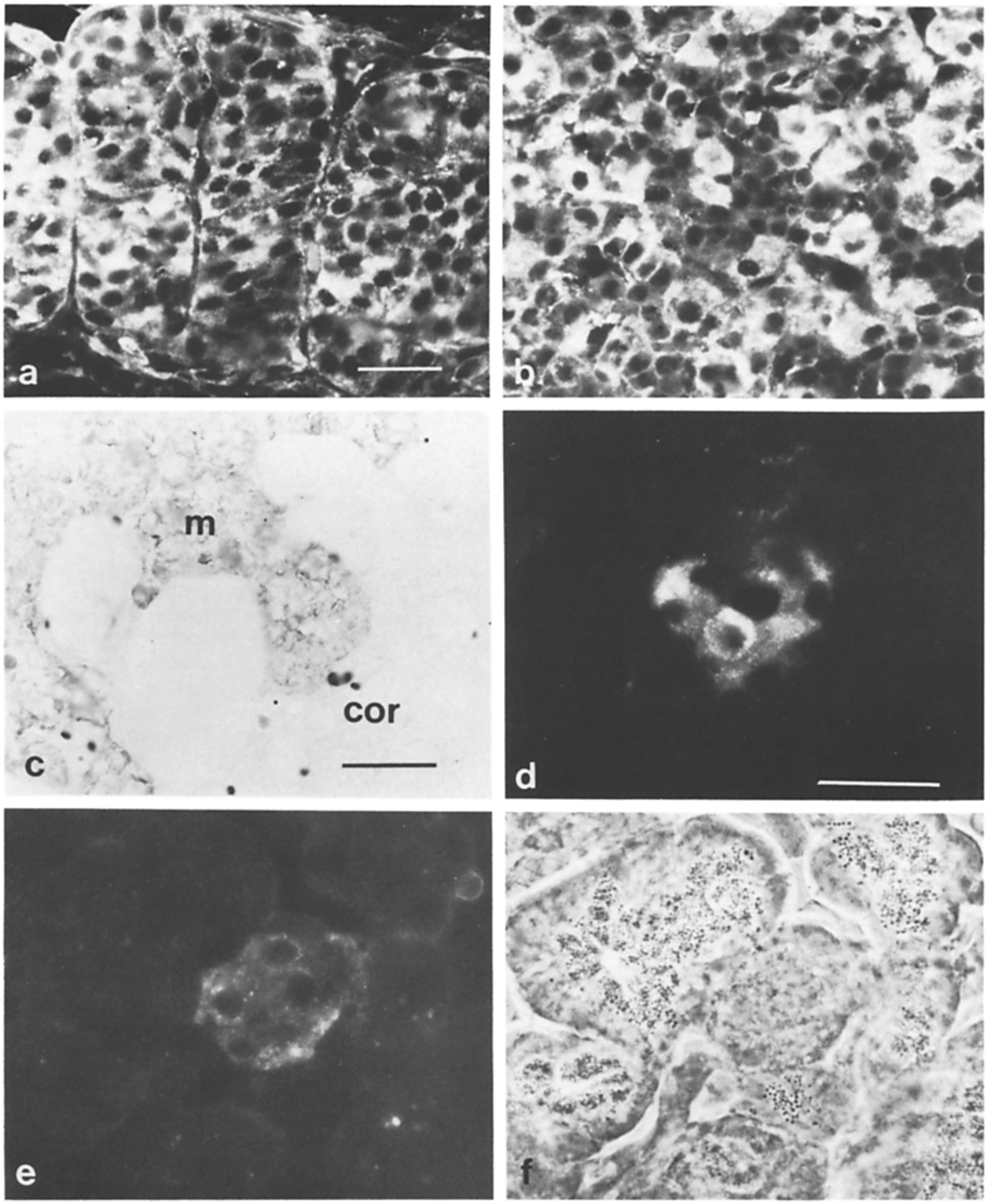


FIGURE 6 Binding to endocrine cells of antibody to the SV2 antigen. (a) Immunofluorescence staining of the rat intermediate pituitary with monoclonal antibody to SV2. (*b*) Immunofluorescence of the rat anterior pituitary. (*c*) Immunoperoxidase staining of the adrenal medulla (*m*). The cortical cells (*cor*) are unstained. (*d* and *e*) Immunofluorescence staining of rat pancreas. Only the islet cells are fluorescent after incubation with antibody to SV2. (*f*) Phase picture corresponding to fluorescent section shown in *e*. Note that the cells that are not fluorescent in *e* contain many phase dense granules, a characteristic of the exocrine cells in the pancreas. Bars: (a and *b*) 25 μm; (c) 50 μm; (*d–f*) 25 μm. (a and *b*) × 560. (c) × 300. (*d–f*) × 800.

branch electric organ that, by immunofluorescence and immunoblotting techniques, was also present in a wide variety of nerve terminals in the central and peripheral nervous system and in several types of endocrine, but not exocrine, cells.

We believe for several reasons that the antigen recognized by the anti-SV2 monoclonal antibody is glycosylated. First, the mobility of the protein in a variety of tissues and species in SDS PAGE was heterogeneous (Fig. 9). Second, when PC12 cells were grown in the presence of tunicamycin, a drug that

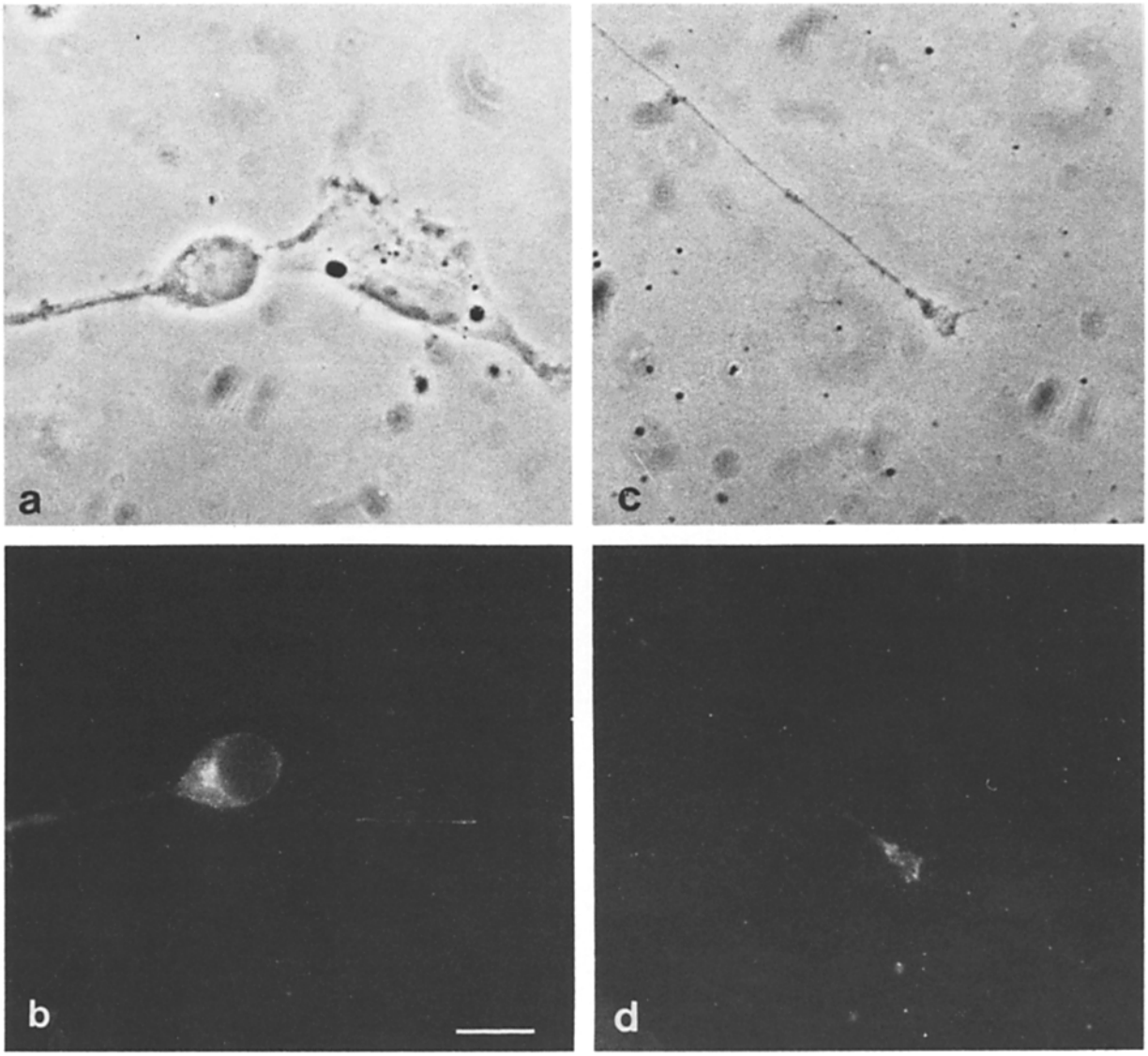


FIGURE 7 Immunofluorescent staining of dissociated sympathetic neurons in culture with monoclonal antibody to the SV2 antigen. (*a*) Phase picture of a neuronal cell body (*left*) and an adjacent non-neuronal cell. (*b*) Corresponding fluorescence picture. Only the neuron shows detectable immunofluorescence, which is localized to the perinuclear region of the cytoplasm. (*c*) Phase picture of neuronal growth cone. (*d*) Fluorescent staining of the growth cone shown in c. Bar, 10 μm. × 1,100.

blocks the addition of asparagine-linked sugars, the apparent molecular weight of the protein in SDS PAGE was reduced by ~30,000 (Fig. 10). Furthermore, the short and long term labeling experiments showed that the protein migrated as a single band with an M_r of 64,000 after short term labeling and appeared as a higher molecular weight form after longer labeling periods (Fig. 10, lanes *a* and *b*). The slightly higher molecular weight of this precursor form of the SV2 protein as compared with the molecular weight of the protein synthesized in the presence of tunicamycin (Fig. 10, lane *c*) is probably due to co-transational glycosylation of the precursor protein (19).

Because glycosylation of most, and probably all, proteins occurs on the luminal side of the membrane, and the antibodies to SV2 bound to the cytoplasmic side of the vesicle membrane (Figs. 2 and 3), we assume that the SV2 glycoprotein spans the vesicle membrane. Furthermore, the antigen is recovered in the detergent phase after solubilization of bovine brain synaptosomes with Triton X-114 (C. K. Kassenbrock, unpublished observations), as is characteristic of integral membrane proteins (3). It should therefore also be possible to generate antibodies to the luminal domain. Such antibodies would be of great value in determining the fate of the glycoprotein after it is externalized by exocytosis. They also would facilitate the selection of variant strains of cells that lack the protein.

Comparison of the mobilities of the SV2 glycoprotein from endocrine and neural sources suggests that the protein in endocrine secretory granules differs slightly from that in neuronal synaptic vesicles. Although there was some variation in the SV2 antigen in brain homogenates from different species, the differences between the electrophoretic mobility in bovine, chicken, mouse, and rabbit brain were slight as compared with the difference between the average of the brain samples and that for three endocrine cell types: PC12 cells, AtT-20 cells, and bovine adrenal chromaffin cells (Fig. 9). Furthermore, a direct comparison of bovine brain (Fig. 9, lane *g*) and bovine adrenal chromaffin cells (Fig. 9, lane *h*) demon-

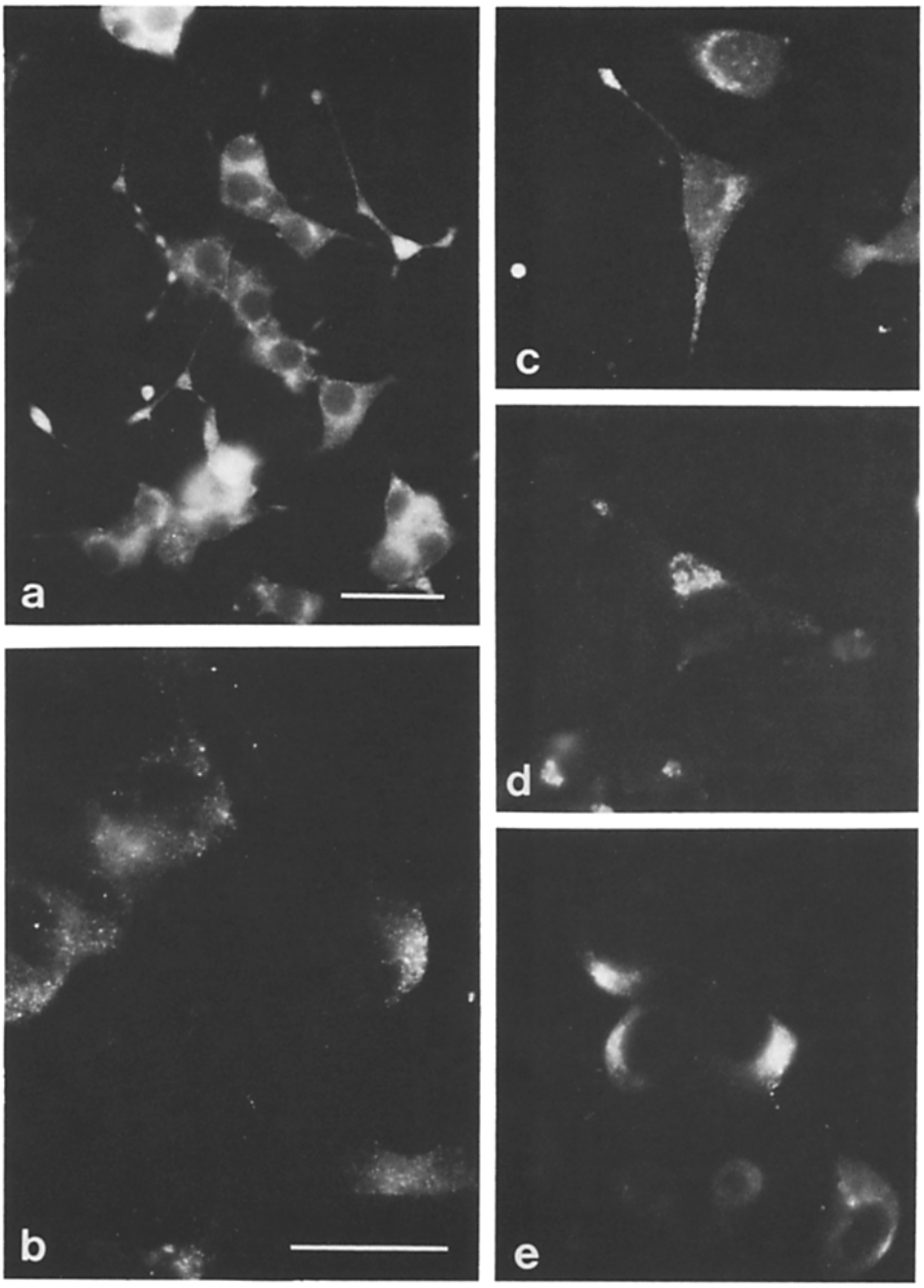
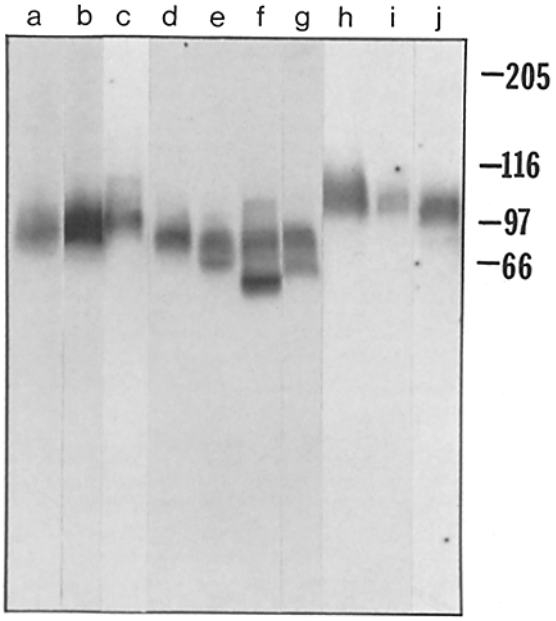


FIGURE 8 Indirect immunofluorescence of endocrine cell lines with monoclonal antibody to SV2 antigen. (*a*) Pheochromocytoma (PC12) cells; (*b*) HIT cells; (*c*) AtT-20 cells stained with mAb to SV2; (*d*) AtT-20 cells stained with affinity-purified antiserum to ACTH; (*e*) GH3 cells. (*a*, *c*, and *d*) Bar, 25 μm. × 560. (*b* and *e*) Bar, 25 μm. × 840.

strates that the difference cannot be explained by species variation alone. Alternatively, the variation in M_r of the SV2 antigen may be correlated with central and peripheral nervous system. The difference in molecular weight of the SV2 antigen in different tissues and species is probably due to the degree of glycosylation of the same polypeptide chain, as is the case with NCAM, a glycoprotein involved in neuronal adhesion. This glycoprotein has different molecular weights in embryonic chick brain and retina, but removal of sialic acid with neuraminidase converts both forms of the molecule to the same molecular weight (17). The possibility has not been eliminated, however, that the apparent difference in M_r of the SV2 antigen in different tissues is due not to glycosylation but to differences in the polypeptide sequence or length.

Only one other membrane protein has been found in both synaptic vesicles and endocrine secretory granules. The protein, described by Matthew et al. (31) has a molecular weight of 65,000 in SDS PAGE. The 65,000-D protein has a distribution in rat nervous system and endocrine tissue that is very similar to that described here for the SV2 glycoprotein. Unlike the SV2 antigen, however, the protein described by Matthew et al. (31) could not be detected in electric organ synaptic vesicles (30) and its molecular weight is the same in all species of brains. The two proteins also appear to be in different populations of bovine brain coated vesicles (33). Synaptic terminals throughout the central and peripheral nervous system also contain a third protein, synapsin I (11). Unlike the 65,000-D protein and the SV2 glycoprotein, synapsin I is a peripheral membrane protein and is not detectable in the adrenal medulla, which suggests that it is associated only with neuronal synaptic vesicles and not with endocrine secretory granules (11).

The absence of the SV2 antigen from exocrine cells could reflect a function unique to secretion from neural and endo-



FIGURES 9 Electrophoretic mobility of SV2 antigen in various species and tissues. Membranes or vesicles were prepared from various tissues, subjected to SDS PAGE, transferred to nitrocellulose, and visualized by blotting with mAb to SV2 and radioactive goat anti-mouse IgG. Approximately 20–60 μg of protein (except lane *c*, 1 μg of purified synaptic vesicles) was loaded per lane, and two different exposures of the same autoradiogram were made to equalize the density of bands. *Lanes:* (*a*) electromotor nucleus (*Discopyge ommata*); (*b*) brain (*Discopyge ommata*); (*c*) synaptic vesicles (*Discopyge ommata*); (*d*) chicken brain membranes; (*e*) rabbit brain membranes; (*f*) mouse brain membranes; (*g*) bovine brain synaptosomes; (*h*) chromaffin granules (bovine); (*i*) PC12 membranes (rat pheochromocytoma); and (*j*) AtT-20 membranes (mouse cell line).

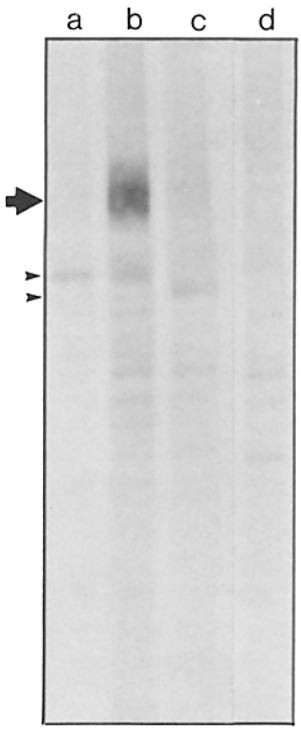


FIGURE 10 Immunoprecipitation of [^{35}S]methionine-labeled SV2 antigen from PC12 cells. Lane *a*, PC12 cells were labeled for 45 min, extracted immediately, and immunoprecipitated with SV2 immunobeads. The labeled material immunoprecipitated by monoclonal antibody to SV2 appeared as a narrow band at M_r 64,000 (upper arrowhead). Lane *b*, PC12 cells were labeled for 5 h and chased for 1 h in the absence of [^{35}S]methionine but with normal amounts of cold methionine. The immunoprecipitated material shows an additional band, which runs more slowly (M_r ~110,000) and has a greater range of electrophoretic mobility (arrow). Lane *c*, PC12 cells were pretreated with tunicamycin and labeled for 5 h with [^{35}S]methionine. The material immunoprecipitated by SV2 immonobeads from these cells runs as a single band of M_r 62,000 (lower arrowhead). Lane *d*, control. PC12 cells were labeled as in *b* but immunoprecipitated with SV1 immunobeads.

crine cells. Alternatively, the protein and its function may be shared by both types of secretory granules, but the antigenic determinant may not be conserved in exocrine cells. Because of the similarities in the mechanisms of secretion in endocrine and exocrine cells, it has been suggested that a common secretory stem cell gave rise to exocrine cells and a neural-endocrine precursor cell. According to this hypothesis, neurons and endocrine cells then differentiated from each other (25). Several lines of evidence suggest that endocrine and neural cells are more closely related to each other than to exocrine cells. Both neurons and endocrine cells secrete many of the same peptides and hormones (23, 25); at least two secretory vesicle membrane proteins (the 65,000-D protein and the SV2 glycoprotein) are common to both types of cells but absent from exocrine cells (reference 31 and this paper); and neuron-specific enolase, previously thought to be specific for neurons, has been found in a wide variety of the endocrine cells but not exocrine cells (2, 36). If endocrine and neuronal cells are closely related by evolution, the absence of the SV2 antigenic site in exocrine cells could result from tissue specific expression of the protein rather than from a basic difference in exocytotic mechanisms.

The widespread distribution of the SV2 antigen in tissue ranging from the elasmobranch to the mammalian brain, and its presence in all neuronal and endocrine tissue we examined, suggests a conserved function for the SV2 glycoprotein. The conserved domain is on the portion of the molecule that faces the cytoplasm. One explanation for a conserved domain on the cytoplasmic surface of secretory vesicles is that it allows interaction with another conserved molecule, for example, a component of the cytoskeleton or the plasma membrane. The existence of such interactions might be testable by microinjection, reconstitution, or a mutational approach.

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