A Nerve Terminal Anchorage Protein from Electric Organ

S. S. Carlson, P. Caroni, and R. B. Kelly

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143.
Dr. Carlson's present address is Department of Physiology and Biophysics, University of Washington, Seattle, Washington 98195.
Dr. Caroni's present address is Hirforschungs-Institut, Universitat Zurich, August Forel Strasse 1, Zurich, Switzerland.

Abstract. The nerve terminal and the postsynaptic receptor-containing membranes of the electric organ are both linked to the basal lamina that runs between them. We have identified an extracellular matrix protein whose physical properties suggest it anchors the nerve terminal to the basal lamina. The protein was identified because it shares an epitope with a proteoglycan component of electric organ synaptic vesicles. It too behaves like a proteoglycan. It is solubilized with difficulty from extracellular matrix fractions, elutes from DEAE Sephacel at pH 4.9 only at high ionic strength, and binds to a laminin affinity column from which it can be eluted with heparin. Under denaturing conditions it sediments rapidly and has a large excluded volume although it can be included in Sephacryl S-1000 columns. This large, highly charged extracellular matrix molecule can be readily reconstituted into liposomes consistent with the presence of a hydrophobic tail. By immunoelectron microscopy the antigen is found both in synaptic vesicles and on the plasma membrane of the nerve terminal. Since this is the first protein described that links the nerve terminal membrane to the extracellular matrix, we propose calling it terminal anchorage protein one (TAP-1).

THE ability of a neuron to make a precise synaptic connection to a target neuron or organ gives rise to the unique information processing power of the nervous system. Although a molecular bridge of some sort must exist between nerve ending and target, identifying and isolating the molecules has proven a difficult task. An unexpected finding suggested that at least in the peripheral system, the molecules specifying synaptic connections should be found in extracellular matrix (ECM).[1] Using regeneration of the nerve–muscle synapse, Sanes et al. (1978) have shown that precise targeting of the regenerating nerve to its original site occurs in the absence of the muscle cell target as long as the original basal lamina of the muscle remains. The molecules that specify the precise regeneration are unknown, as is their cell of origin. Antibodies and lectins that bind specifically to ECM components of the synaptic region have been described (for review see Sanes, 1983) but identification and purification of the unique extracellular molecules is a formidable task given the minute quantities of material available.

The electric organ of marine rays, an organ embryologically related to muscle, has been a rich source of the molecular components of synapses including extracellular factors involved in acetylcholine receptor and acetylcholinesterase clustering (Fallon et al., 1985; Wallace et al., 1985). We have described an antigen, the SV4 antigen, that is enriched in ECM of electric organ synaptic junctions and is transported there by the nerve that innervates the electric organ (Caroni et al., 1985). In this paper, we have used a monoclonal antibody to the SV4 antigen to isolate from electric fish ECM fractions (Godfrey et al., 1984) molecules bearing this antigen. Only one major component can be solubilized from the ECM. It is found to be a highly charged molecule of very large size, with a membrane-associating domain. By immunoelectron microscopy it is found in association with the outside of the nerve terminal but not elsewhere in the electric organ. These properties are those that would be expected of a nerve terminal anchorage protein (TAP).

1. *Abbreviations used in this paper*: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]l-propane-sulfonate; ECM, extracellular matrix; TAP, terminal anchorage protein.

Materials and Methods

Materials

$Na^{125}I$ was obtained from Amersham Corp. (Arlington Heights, IL), iodogen was purchased from Pierce Chemical Co. (Rockford, IL), centricons from Amicon Corp. (Danvers, MA), Triton X-100 from Boehringer Mannheim Biochemicals (Indianapolis, IN), ultrapure urea from Schwarz/Mann Div. (Spring Valley, NY), 10-nM gold goat anti-mouse IgG from Jansen Pharmaceutica, Structure Probe, Inc. (West Chester, PA), and Lowicryl K4M from Polysciences, Inc. (Warrington, PA). DEAE Sephacel, Sephacryl S-1000, and Sephadex G-25 were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). BSA, 3-[(3-cholamidopropyl)-dimethylammonio]l-propane sulfonate (CHAPS), Hepes, SDS, Nonidet P-40, heparin, sucrose, diisopropylfluorophosphate, iodoacetamide, pepstatin, leupeptin, chymostatin, and guanidine-HCl were purchased from Sigma Chemical Co. (St. Louis, MO). The laminin bound to Affi-Gel 10 was a gift from the laboratory of Dr. L. F. Reichardt at University of California, San Francisco. The electric ray, *Discopyge ommata*, was obtained from Salt Water Aquariums (San Francisco, CA).

Immunogold Electron Microscopy

Discopyge ommata was anesthetized and perfused as previously described for *N. brasiliensis* (Carlson et al., 1978). Before removing the electric organs, the fish were also perfused with 200 ml of 0.8 M NaCl, 10 mM Na cacodylate, pH 7.4, and then 200 ml of 0.1 M Na cacodylate, 70 mM NaCl, 4% paraformaldehyde, 0.1% glutaraldehyde, pH 7.4. A second fish was not perfused, but small pieces of electric organ were fixed by immersion in the paraformaldehyde-glutaraldehyde solution for 2 h. The tissue samples were then processed as described in Valentino et al. (1985) for immunogold electron microscopy with Lowicryl K4M. Monoclonal supernatants were used undiluted. The two fish gave essentially the same results and both were used in the analysis.

Morphometric Studies

Grids were examined and photographed using a JEOL 100B electron microscope. The lengths of membranes were made from enlarged photographic prints using a digitizer pad. Areas of the micrographs were measured by cutting out and weighing photocopies of the desired regions.

Dot Blot Assays (Immunoblots)

Dot blot assays on nitrocellulose (BA85; Schleicher & Schuell, Inc., Keene, NH) to detect SV4 antigenicity were done as previously described (Carlson and Kelly, 1983) except where the antigen was in a denaturing solution other than SDS. 4 M guanidine-HCl/CHAPS containing samples were diluted 1:200 in spotting buffer (2% SDS, 0.2 M NaCl, 5 mM Hepes, 10 mM beta-mercaptoethanol, pH 7.0) before application to the nitrocellulose; 8 M urea detergent-containing samples were diluted 20-fold before application. In some cases, especially after protease digestion, samples were applied to either DEAE nitrocellulose (NA45; Schleicher & Schuell, Inc.) or DEAE paper (DE81; Whatman Inc., Clifton, NJ). Here the spotting buffer was 8 M urea, 0.1 M NaCl, 50 mM sodium acetate, 0.2% Nonidet P-40, pH 4.9. The binding of the monoclonal antibody to filters (50-fold dilution of culture supernatant) was detected using ^{125} I-affinity-purified goat anti-mouse IgG, H & L chain specific (Cappel Scientific Division, CooperBiomedical, Inc., Malvern, PA). The bound radioactivity was detected by counting the filters in a gamma counter or by autoradiography with an intensifying screen (Cronex Lightning-Plus; DuPont Co., Wilmington, DE). In the latter case the signal was quantitated by densitometry of the exposed x-ray film (Kodak, XAR-5) with a Zeineh Soft Laser scanning densitometer, model SL-504-XL (Biomed Instruments, Inc., Fullerton, CA).

Preparation of the ECM Fraction

The procedure was a small modification of that of Godfrey et al. (1984). All manipulations were done at 4°C. The crushed frozen electric organ from *D. ommata* was prepared as described by Carlson et al. (1978). 300 g of the crushed tissue was homogenized with a Waring blender for 2 min in 450 ml of 0.4 M NaCl, 10 mM EGTA, 10 mM Hepes, 0.02% sodium azide, pH 7.4, and spun at 12,500 *g* for 30 min. The supernatant was discarded and the pellet re-homogenized in 200 ml of 0.5 M NaCl, 10 mM EDTA, 10 mM Tris, 17 mM iodoacetamide, 0.125 μl/ml diisopropylfluorophosphate, 0.025 mg/ml pepstatin, chymostatin, and leupeptin (all three added to the homogenate at 5 mg/ml in dimethyl sulfoxide), pH 7.5. The protease inhibitors were added just before the homogenization. The homogenate was spun at 12,500 *g* for 30 min. The supernatant was discarded and the pellet re-homogenized in 300 ml of 10 mM Tris, pH 7.5. The homogenate was spun at 31,000 *g* for 30 min. The supernatant was discarded and the pellet mixed with 350 ml of 20 mM Tris, 3% Nonidet P-40, pH 10.5, using a magnetic stirring motor for 2 h. The extract was spun at 31,000 *g* for 30 min. The supernatant was discarded and the pellet frozen for further use. No difference in the antigen was found whether the protease inhibitors were added at the first or second homogenization.

Solubilization of the SV4 Antigen from the ECM Fraction

Two solubilization conditions were used. For SDS solubilization, each gram of ECM pellet was mixed with 10 ml of 5% SDS in 0.12 M Hepes, 1% beta-mercaptoethanol, pH 6.8, and boiled for 4 min. For guanidine-HCl extraction, each gram of ECM pellet was rotated 18 h at 4°C in 5 ml of 4 M guanidine-HCl, 1 mM EDTA, 10 mM beta-mercaptoethanol, 50 mM sodium acetate, pH 5.8, and either 0.2% Nonidet P-40, 50 mM sodium acetate, pH 5.8, or 2% CHAPS, 50 mM Hepes, pH 7.5. Samples were further homogenized in a Polytron (Brinkmann Instruments Co., Westbury, NY) then centrifuged at 20,000 *g* for 20 min. Recovery of SV4 antigen in the supernatant was about four times higher for homogenization in guanidine-HCl than in SDS even with boiling.

Sedimentation Velocity of the SV4 Antigens in SDS

Analytical sedimentation velocity ultracentrifugation of the SDS-solubilized ECM in sucrose gradients containing SDS was performed with a SW-60 rotor (Beckman Instruments, Inc., Palo Alto, CA) as previously described (Carlson and Kelly, 1983). For preparative sedimentation velocity, a 2.5-ml sample was layered on a 36-ml sucrose gradient and spun in an SW-28 rotor for 19.3 h. 2-ml fractions were collected. To remove SDS from samples, they were dialyzed exhaustively against 0.2% SDS, 8 mM Hepes, pH 7.0, lyophilized, and redissolved in distilled water. To precipitate the SDS, KCl was added to a final concentration of 0.2 M, incubated on ice for 30 min, and spun at 15,600 *g* for 10 min. The resulting pellet was extracted three times with 5 ml of HCl/acetone (165 μl of 3 M HCl/5 ml acetone) using a 15,600 *g* spin to separate supernatant and pellet. The pellet was dried with a Speed-Vac (Savant Instruments, Inc., Hicksville, NY).

DEAE-Sephacel Chromatography of Solubilized ECM Components

Chromatography on DEAE-Sephacel at pH 5.0 is commonly used to separate highly charged anionic molecules such as proteoglycans. To keep them in solution, it is necessary to run the columns in 8 M urea. Samples were made up in 8 M urea, 0.11 M NaCl, 0.2% Nonidet P-40, 50 mM sodium acetate, pH 4.9, centrifuged to remove insoluble material (20,000 *g*, 20 min), and applied to a 2-ml DEAE Sephacel column which was equilibrated with the same buffer. After washing with three column volumes, proteins were eluted with a linear gradient of NaCl, 0.11-1.4 M, in the same urea buffer. The elution volume was twelve to twenty times the column volume. Columns were run at room temperature.

Chromatography of Solubilized ECM on Sephacryl S-1000

ECM material solubilized in guanidine-HCl and CHAPS was applied to a 120-ml column equilibrated with 4 M guanidine-HCl, 0.2% CHAPS, 50 mM Hepes, pH 7.4. Chromatography was carried out at a flow rate of 24 ml/h at room temperature. To assay for protein, column fractions were concentrated to 150 μl and the 4 M guanidine-HCl in the column buffer replaced with 8 M urea by repeated concentrations with a Centricon-30. This was done to avoid the incompatibility of the 4 M guanidine-HCl and the protein assay of Schaffer and Weissmann (1973).

Pronase Digestion of ECM Fraction and Chromatography on Sephacryl S-1000

0.25 g of ECM pellet was resuspended in 1.5 ml of 50 mM Hepes, 0.83% Nonidet P-40, pH 7.5, which contained 20 mg/ml pronase (No. 53702, Calbiochem-Behring Corp., San Diego, CA) and was incubated for 19 h at room temperature. The digest was then spun at 15,600 for 15 min and the supernatant heated in a boiling water bath for 5 min to inactivate the pronase. 1.2 ml of this supernatant was mixed with 6 M guanidine-HCl, 0.2% Nonidet P-40, 50 mM Hepes, 24 mM beta-mercaptoethanol, pH 7.5, and applied to a 120-ml Sephacryl S-1000 column. The conditions of the chromatography were as previously described.

Preparation of Iodinated SV4 Antigen from ECM

Peak fractions from the Sephacel S-1000 chromatography of ECM were pooled and the guanidine-HCl/CHAPS buffer exchanged for 6 M urea, 0.2% CHAPS, 50 mM Hepes, pH 7.0, by repeated concentrations with a Centricon-30. The resulting 100 μl of solution was mixed with 1 mCi of Na^{125}I and iodinated with iodogen (Salacinski et al., 1981). The unbound ^{125}I was separated from bound radioactivity (4×10^7 cpm) by spin dialysis with Sephadex G-25 fine swollen in the urea/CHAPS buffer. The iodinated material was further purified by velocity sedimentation in SDS sucrose gradients as described previously. To evaluate the purity of this ^{125}I-SV4 antigen, a fraction was diluted to 700 μl of 8 M urea, 50 mM NaCl, 50 mM sodium acetate, 0.2% Nonidet P-40, pH 4.9, and analyzed on a DEAE-Sephacel column. The radioactivity eluted at the expected position.

Binding to Laminin Affigel

Iodinated SV4 antigen was diluted 100-fold with 40 mM NaCl, 10 mM Hepes, 0.2% Triton X-100, pH 7.0, and loaded on a 0.6-ml column of laminin covalently bound to Affi-gel 10 (Bio-Rad Laboratories, Richmond, CA). The flow rate was 29 ml/h and 0.6-ml fractions were collected. The column was washed with 12 ml of the same buffer and then eluted with the same buffer containing 10 mg/ml heparin.

To verify that the eluted radioactivity was indeed the SV4 antigen, aliquots of the peak fractions were immunoprecipitated with immunobeads (Bio-Rad Laboratories, Richmond, CA) to which the monoclonal antibody directed against SV4 was bound. Rabbit anti-mouse Immunobeads (20 mg) washed with 0.4 M NaCl, 10 mM Hepes, 1% BSA, pH 7.0, were resuspended in 5 ml of monoclonal supernatant and mixed overnight by rotation. The beads were washed as before and resuspended in 0.4 M NaCl, 10 mM Hepes, 1% Triton X-100, 0.1% SDS, pH 7.0 (precipitation buffer). To immunoprecipitate fractions, aliquots (containing <0.05% SDS) were mixed with an equal volume of antibody-coupled immunobeads (1 mg/ml) and rotated overnight. Samples were underlayed with a pad of 7% Ficoll in precipitation buffer and spun for 10 min at 15,600 *g*. The bead pellet was then washed three times with precipitation buffer. An identical set of immunoprecipitations was performed with beads coupled to a monoclonal antibody that does not recognize any ECM component. All procedures in binding antibody to the beads and the immunoprecipitations were done at 4°C.

Incorporation into Liposomes

To incorporate proteoglycans into liposomes by the procedure of Brunner et al. (1978), the detergent Nonidet P-40 in the DEAE-Sephacel-purified proteoglycans first had to be exchanged for cholate. In brief, peak fractions from DEAE-Sephacel columns were diluted fivefold in 8 M urea, 20 mM Tris, pH 7.4, to reduce the ionic strength, then adsorbed to a small (2 ml total volume) DEAE-Sephacel column. After washing with 2% cholate, 0.1 M NaCl, pH 7.4, 20 mM Tris, 8 M urea, the proteoglycan was eluted with the same buffer containing 3 M NaCl. 1 ml of the eluted fraction was added to a tube containing 10 mg of egg phosphatidylcholine and trace amounts of ^{3}H-cholesterol (4 × 10^6 cpm, 5 μg; New England Nuclear, Boston, MA) dried under a stream of nitrogen. The resulting solution was fractionated on a Sephadex G-50 column (1.4 × 40 cm) equilibrated with 0.1 M NaCl, 20 mM Tris, pH 7.4, and 45% sucrose. Liposomes were eluted in the void volume.

To show association of proteoglycan and liposomes, a linear density gradient of 5–35% sucrose (10 ml, 0.1 M NaCl, 20 mM Tris, pH 7.4) was layered onto 2 ml of the liposome suspension (in 45% sucrose) in a Beckman SW41 centrifuge tube. The samples were centrifuged at 35,000 rpm at 10°C for 12 h, and fractions collected from the bottom. Densities of gradient fractions were measured by refractometry.

To separate proteoglycan-containing liposomes on DEAE-Sephacel, columns of 2 ml were washed with 0.05 M NaCl, 20 mM Tris, pH 5.0. The liposomes from the G-50 column were passed through the column, the column washed with 5 ml of the same buffer, then the proteoglycans eluted with a 0.05–1.2 M NaCl gradient. To separate free proteoglycans from those in liposomes, the peak fractions were layered on a 5–60% sucrose density gradient (0.1 M NaCl, 20 mM Tris, pH 7.4) in a Beckman SW41 centrifuge tube and centrifuged for 16 h at 35,000 rpm (10°C). To remove the external proteoglycan with pronase, an aliquot was incubated at room temperature for 2 h in 20 μg/ml pronase (Calbiochem-Behring Corp.).

Phospholipid was estimated in all samples by scintillation counting of ^{3}H-cholesterol and antigenicity by dot blot assay using antibodies to the SV4 site for ECM proteoglycan and anti-SV1 for vesicle proteoglycan (Caroni et al., 1985).

Other Techniques

Synaptic vesicles from the electric organ of *D. ommata* were purified by the methods of Carlson et al. (1978). Protein concentration was measured with the filter binding assay of Schaffer and Weissmann (1973).

Results

We have shown earlier that an antigenic determinant present on a synaptic vesicle proteoglycan is also present at high specific activity in the ECM (Caroni et al., 1985). To test whether the antigenic site was on the same molecule in vesicles and ECM, antigenic molecules from the two sources were compared. The antigenic molecule was solubilized from the ECM fraction in 1% SDS buffer containing beta-mercaptoethanol and its sedimentation velocity on a sucrose density gradient compared to that solubilized from synaptic vesicles. Note that the two gradients were centrifuged for different times. As can be seen in Fig. 1, the antigens from both sources sediment as single peaks indicating that they are monodisperse. The uncorrected *s* values were 11.2 for the ECM form and 2.2 for the synaptic vesicle proteoglycan. Since the sedimentation velocities of the two molecules differed by a factor of 5, even under severe denaturing conditions they must differ by size, density, or both.

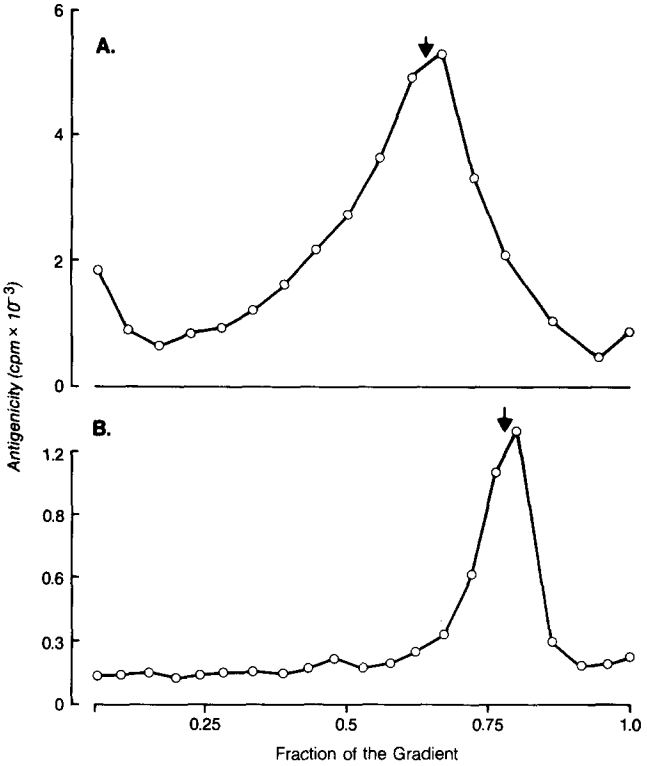


Figure 1. Sedimentation velocity of the ECM and synaptic vesicle SV4 antigens in SDS. *A* shows the sedimentation of the SV4 antigen solubilized from the ECM (7 mg ECM pellet) by SDS and run in a 5–20% sucrose gradient containing SDS for 2.2 h at 48,000 *g*. *B* shows the sedimentation of the SV4 antigen from synaptic vesicles (26 μg vesicle protein) run under the same conditions for 8.6 h. The arrows mark the positions on the gradients which were used for the calculation of the sedimentation coefficients. Antigenicity in 10 μl of each fraction was measured by dot blotting on nitrocellulose.

To confirm this difference, the two molecules were compared on sizing columns. Using 4 M guanidine-HCl, and 0.2% CHAPS to solubilize the ECM form, over four times more antigen could be solubilized than SDS. Although the vesicle proteoglycan is included (K_{av} = 0.2) in a Sepharose 6B column (Carlson and Kelly, 1983), the ECM antigen was excluded even by a Sepharose 4B column. The ECM form was included in a Sephacryl S-1000 column (Fig. 2), although it eluted before the blue dextran marker which has a size of ~2 × 10^6. By comparison with the profile of protein eluting from the S-1000 column, ECM proteins of this size are quite rare.

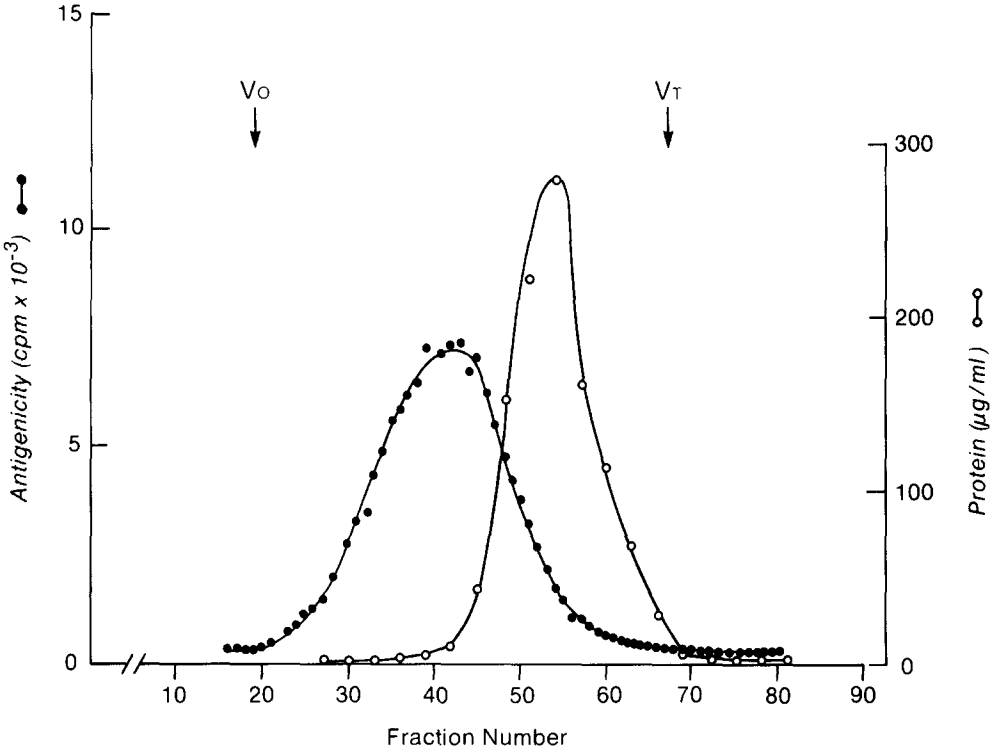


Figure 2. Permeation chromatography on the extracellular matrix SV4 antigen on Sephacel S-1000 in 4 M guanidine-HCl, 0.2% CHAPS. The SV4 antigenicity and protein concentration for selected fractions are shown plotted on the figure. The calculated void volume (V_o), and the total volume (V_T) are indicated on the figure by the labeled arrows. 0.5 g of ECM pellet was solubilized in 2.9 ml of 4 M guanidine-HCl, 50 mM Hepes, 2% CHAPS, 0.36 M beta-mercaptoethanol, pH 7, and applied to a 120-ml Sephacel S-1000 column. The column was equilibrated with the same buffer containing 0.2% CHAPS and no beta-mercaptoethanol. The ordinate gives the antigenicity per 10 µl.

A second characteristic of the synaptic vesicle proteoglycan is its high negative charge (Carlson and Kelly, 1983). As a result of this charge, it elutes from DEAE-Sephacel at 0.46 M salt at pH 5.0 (data not shown). The ECM antigen, isolated by its high sedimentation velocity (Fig. 1) was passed through a DEAE-Sephacel column under similar conditions and its elution conditions were studied. Essentially, all the rapidly sedimenting antigen bound to the DEAE column (Fig. 3) and was only eluted at higher salt concentration (0.7 M). It appears therefore as if by this second criterion, the antigenic molecule in the synaptic vesicle is not identical to that in the ECM, although both are highly negatively charged. Fig. 3 also demonstrates that all of the rapidly sedimenting ECM form is highly negatively charged.

Only a small fraction of the ECM protein solubilized by 4 M guanidine-HCl binds to DEAE-Sephacel and of that very little elutes with the antigen at 0.7 M salt (Fig. 4). The antigenic molecule is therefore a minor ECM component.

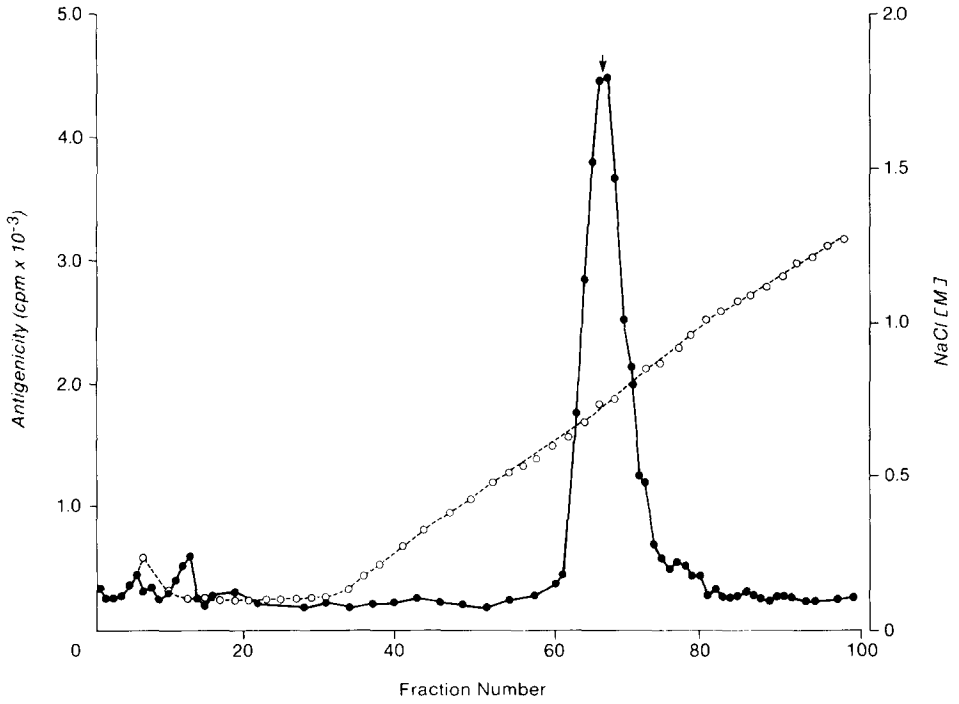


Figure 3. Ion exchange chromatography of the SV4 antigen on DEAE-Sephacel after isolation by sedimentation velocity centrifugation. The SV4 antigenicity (●) and the NaCl concentration (○) of selected fractions are shown plotted on the figure. Approximately 3.0 mg of protein from which the SDS was extracted was redissolved in 2 ml of 8 M urea, 0.2% Nonidet P-40, 0.11 M NaCl, 50 mM Hepes, pH 4.9, and applied to a 2-ml DEAE Sephacel column. The column was washed with 6.5 ml of this same buffer and then eluted with a linear gradient of 36 ml from 0.11 to 1.4 M NaCl in this same buffer solution. The flow rate was 10 ml/h, 0.5-ml fractions were collected, and 5-µl samples were assayed.

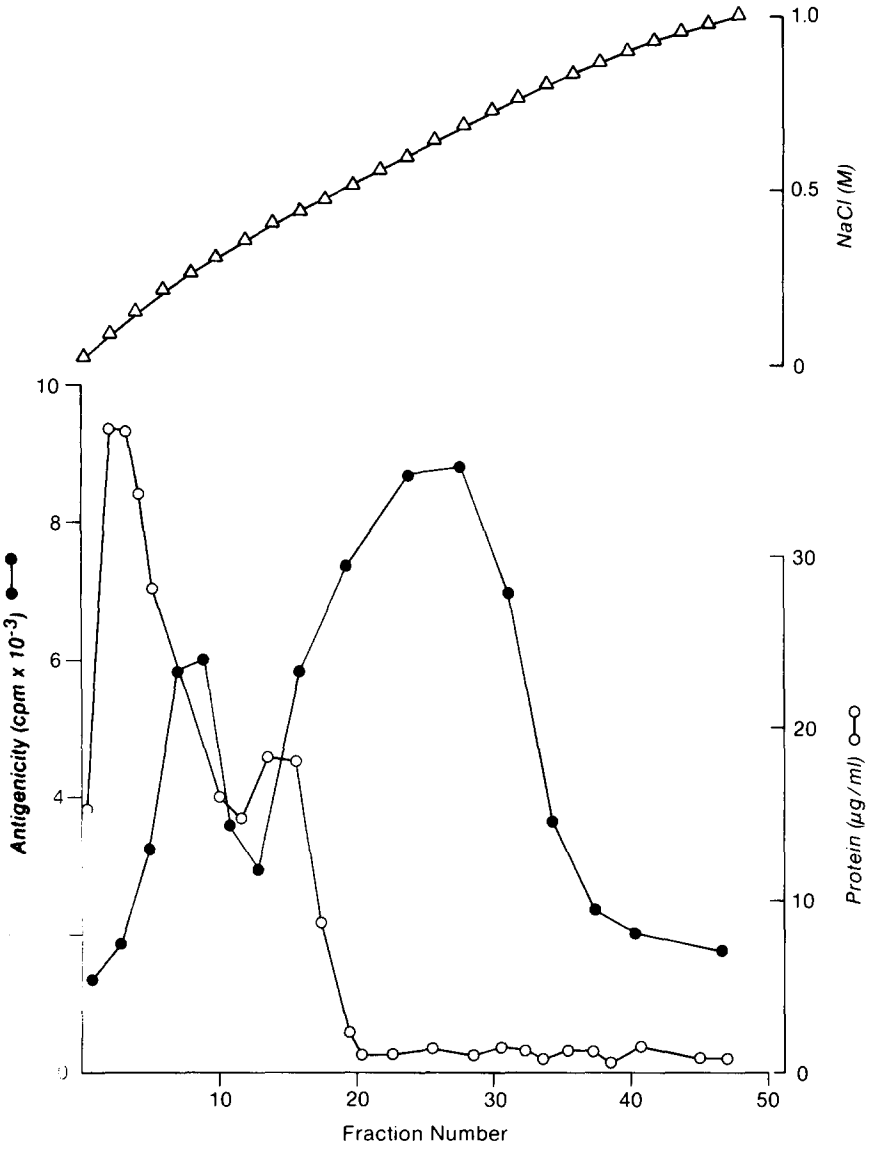


Figure 4. The elution profile of guanidine-HCl solubilized ECM fraction from DEAE-Sephacel. SV4 antigenicity (●) and protein concentration (○) are shown in the figure. About 16 g of ECM pellet was solubilized in 4 M guanidine-HCl, 50 mM sodium acetate, 50 mM EDTA, 0.2% Nonidet P-40, 10 mM beta-mercaptoethanol, pH 5.8. This extraction buffer was exchanged by dialysis with a buffer solution containing 8 M urea, 0.11 M NaCl, 50 mM sodium acetate, 0.2% Nonidet P-40, pH 5.0. The resulting 60 ml of sample was applied to a 12-ml DEAE-Sephacel column, eluted with 150 ml linear salt gradient (0.11–1.4M NaCl; Δ) at a flow rate of 10 ml/h, 2.4-ml fractions were collected, and 1-μl fractions assayed. Only the gradient portion of the elution profile is shown on the figure.

Note that the antigenic molecule does not elute in such a sharp peak in Fig. 4. This is a consistent finding when less pure samples are subject to DEAE chromatography.

Association of the Extracellular Antigen with ECM Components

Dissociation of the SV4 antigen from the ECM fraction requires strong denaturing conditions. The ECM fraction used in these experiments is a detergent-insoluble material that cannot be solubilized by high salt, low salt, or calcium chelators. It is enriched in collagen and in acetylcholine receptor clustering factors (Godfrey et al., 1984). The clustering factor and other loosely attached proteins can be removed by a pH 5.0 wash (Fallon et al., 1985), but the antigen is not disturbed. Boiling in 1% SDS solubilizes 70 times more antigen than non-denaturing detergent (0.4% Triton X-100) while 4 M guanidine-HCl, in the presence of 2% Nonidet P-40, solubilizes 270 times as much.

To explore how the extracellular antigen might be associating with the ECM, we examined its binding to laminin affinity columns. The antigen was extracted from the ECM fraction using guanidine-HCl and chromatographed on Sephacryl S-1000 (Fig. 2) to give partial purification. To allow its detection, the antigen was iodinated with ^{125}I and further purified by velocity sedimentation. At least 63% of the recovered radioactivity eluted at 0.68 M salt on DEAE-Sephacel and 65% could be specifically immunoprecipitated with antibody to SV4.

When the purified iodinated material was passed over a laminin affinity column, 23% of the recovered radioactivity passed through the column without binding, while 77% (Fig. 5) could be eluted with heparin (10 mg/ml). To confirm that the radioactive material was indeed in the antigen, fractions were immunoprecipitated with antibody to SV4. The majority (71%) of the eluted counts were immunoprecipitated with anti-SV4, while control antibody gave no immunoprecipitation. We conclude that the extracellular antigen can associate with molecules such as laminin, presumably via its highly charged domain. The competition with heparin suggests that

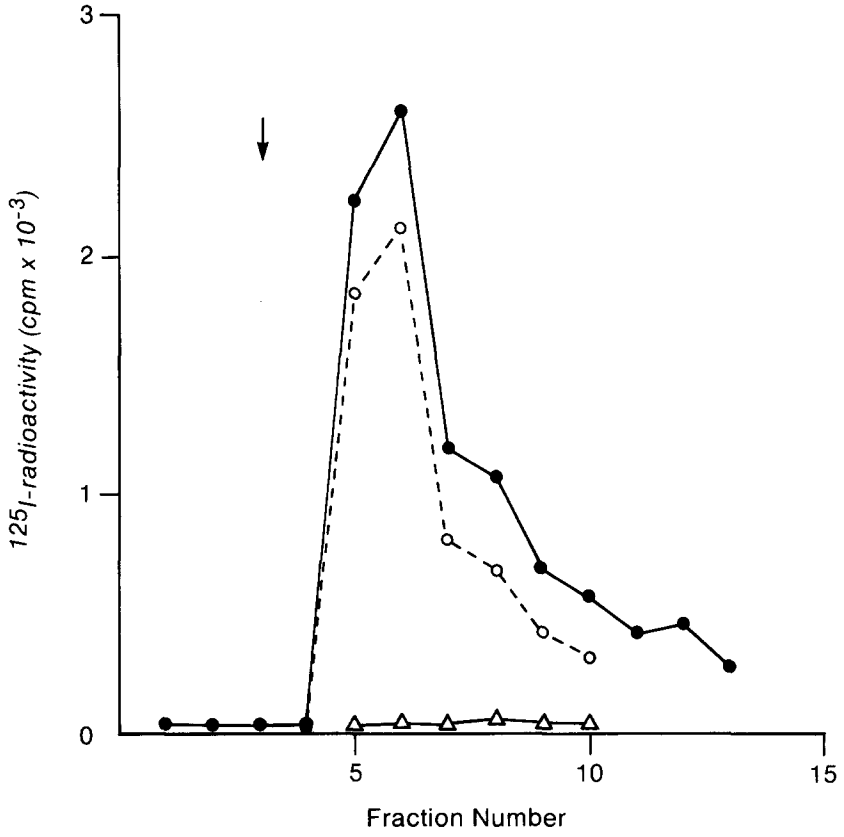


Figure 5. The binding of the ECM-SV4 antigen to immobilized laminin and its elution by heparin. ^{125}I-ECM-SV4 antigen (isolated by chromatography on Sephacryl S-1000 and sedimentation velocity) was applied in 10 ml of column buffer (40 mM NaCl, 10 mM Hepes, 0.2% Triton X-100, pH 7.0) to a 0.6-ml column of laminin covalently bound to affi-gel. The column was then washed with 12 ml of the same buffer. Only about 23% of the radioactivity eluted from the column during these washes. When the radioactivity had dropped to background levels, the laminin-Affi-gel was eluted with column buffer containing 10 mg/ml heparin (as indicated by the arrow on the figure). The flow rate was 29 ml/h and 0.6-ml fractions were collected. Radioactivity was assayed (●). To verify that the label was in antigenic molecules, aliquots (250 μl) of fractions (5–10) were subjected to immunoprecipitation using polyacrylamide beads to which antibody to SV4 antigen was attached (○). The control immunoprecipitations (△) were done using beads to which a nonspecific monoclonal antibody was bound.

the antigenic molecule is a proteoglycan but we have no direct chemical evidence.

Evidence for a Protease-resistant Domain

The molecule carrying the SV4 antigen has pronase-sensitive regions, since exposure to pronase prevents binding to nitrocellulose paper (Caroni et al., 1985). The antigenicity can be recovered in a small, neutral glycopeptide domain. A significant portion of the antigen appears, however, to be associated with a large, pronase-resistant domain which is negatively charged and will bind to DEAE-nitrocellulose paper. To estimate the size of this domain, it was chromatographed on Sephacryl S-1000 and compared with undigested material. 4 M guanidine-HCl was present throughout. The digested material runs more slowly than the intact molecule but nonetheless elutes at the position of the blue dextran marker (Fig. 6). The k_{av} for the untreated material

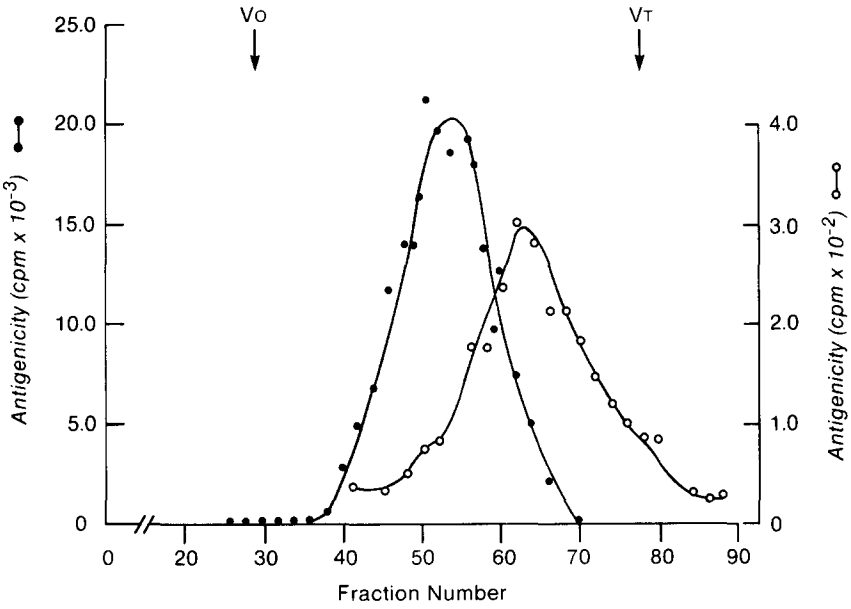


Figure 6. The chromatographic separation of pronase digested and undigested ECM-SV4 antigen on Sephacryl S-1000. The undigested (●) and pronase digested (○) molecules were chromatographed separately on the column, as in Fig. 4. The arrows labeled V_o and V_T show the calculated void volume and the total volume of the column, respectively. Both the undigested ECM pellet (0.25 g) and the pronase-treated ECM pellet (0.18 g) were mixed with guanidine-HCl/CHAPS before application to the column. The antigenicity for the undigested antigen was detected using 2.5–5-μl samples and the dot blot assay on DEAE-nitrocellulose. All these assays gave essentially the same results; the results of the DEAE-nitrocellulose assay are shown. The pronase-resistant antigenicity was measured in 15–30-μl aliquots using the DEAE paper dot blot assay.

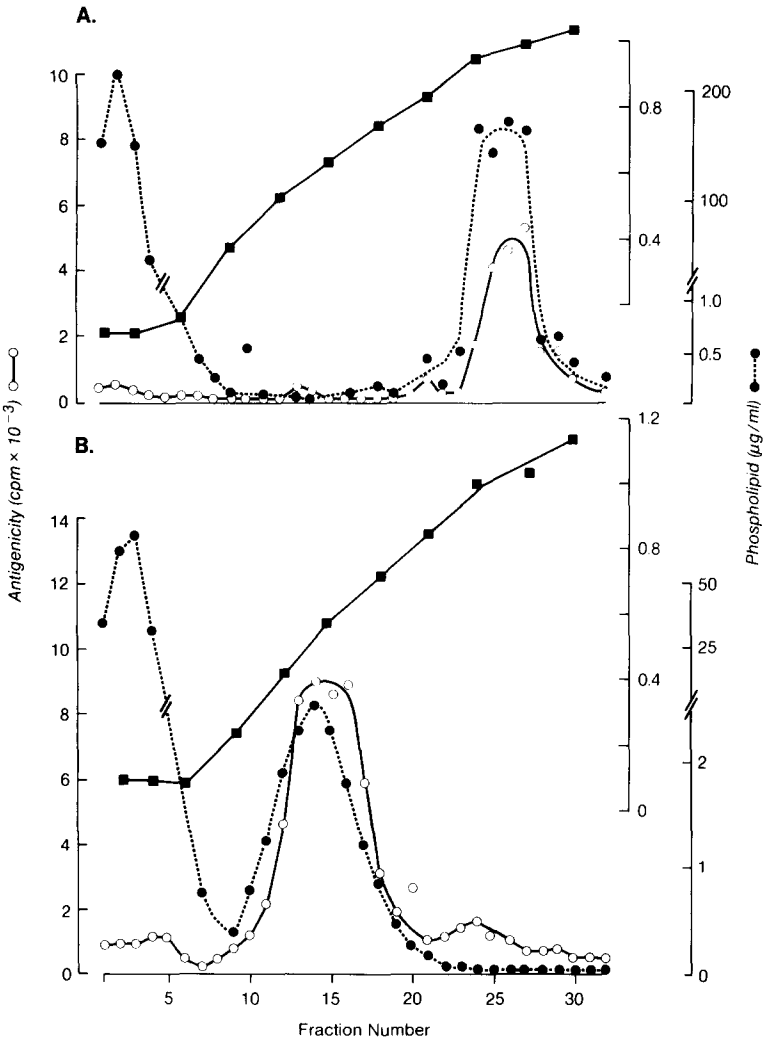


Figure 7. Chromatography of antigen-containing liposomes on DEAE-Sephacel. Liposomes (5-ml volume) prepared by chromatography on a G50 Sephadex column were passed through 2-ml columns of DEAE-Sephadex which was then washed with 0.05 M NaCl, 20 mM Tris, pH 5.0. Most of the phospholipid (●) but little of the antigen passed straight through the column. Antigen (○) eluted by a salt gradient (0.05–1.4 M NaCl, 50 mM Hepes, pH 5.0; ■) was associated with a small peak of phospholipid. Note the change in phospholipid scale. (*A*) Liposomes containing ECM. Synaptic vesicle SV4 antigen was assayed by dot blot on nitrocellulose filters using antibody to SV4. (*B*) Liposomes containing the synaptic vesicle proteoglycan assayed by antibody to SV1.

was 0.52 and for the digested material is 0.69. Although accurate measurements cannot be made, because we do not know to what extent protein and carbohydrate standards are appropriate, the digested material is about one-tenth the size of the intact form. Given the size of the molecule, there may be more than one pronase-resistant domain per polypeptide.

The Extracellular SV4 Antigens Also Have Hydrophobic Domains

Several heparan sulfate proteoglycans have been found that have membrane-associated tails, indicating that they are integral membrane proteins (Höök et al., 1984*a*). If the SV4 antigen had a hydrophobic tail then it could act as a bridge to link membranes and the ECM material. We therefore looked for the presence of hydrophobic tails using established procedures (Kjéllen et al., 1980). The Nonidet P-40 detergent was removed from SV4 antigen purified on a DEAE-Sephacel column by adsorbing it at low ionic strength to a second DEAE-Sephacel column, washing the column with a buffer containing cholate, then eluting batchwise in high ionic strength medium also containing cholate. Excess phosphatidyl choline containing a small amount of ^{3}H-cholesterol as marker was added to the SV4 antigen and unilamellar liposomes obtained by the filtration procedure of Brunner et al. (1978). Proteins that associate with phospholipids float to light density positions on equilibrium centrifugation on sucrose density gradients (Skehel et al., 1982). By this criterion, the majority of the antigenic molecules had hydrophobic domains and came to equilibrium at a density of 1.11 gcm^{-3}. The equilibrium density of the liposomes containing the ECM SV4 antigen (1.10 ± 0.01, four measurements) was always slightly greater than that of free liposomes (1.055 ± 0.009). To confirm that this indeed represented incorporation into liposomes, liposomes were solubilized by adding the detergent deoxycholate before centrifugation. The antigens did not float upwards (data not shown).

The presence of a phospholipid-binding region was confirmed by fractionating liposomes on DEAE-Sephacel. The majority of the phospholipid passed through the column (Fig. 7). A small peak, however, eluted with the SV4 antigen when the salt concentration was raised. In contrast, liposomes containing the synaptic vesicle proteoglycan (Carlson and Kelly, 1983) eluted at a characteristically lower ionic strength (0.47 M NaCl). When the antigen-associated liposomes purified on a DEAE-Sephacel column were centrifuged on a sucrose density gradient (Fig. 8), the majority (64%) of the antigen came to equilibrium at a density close

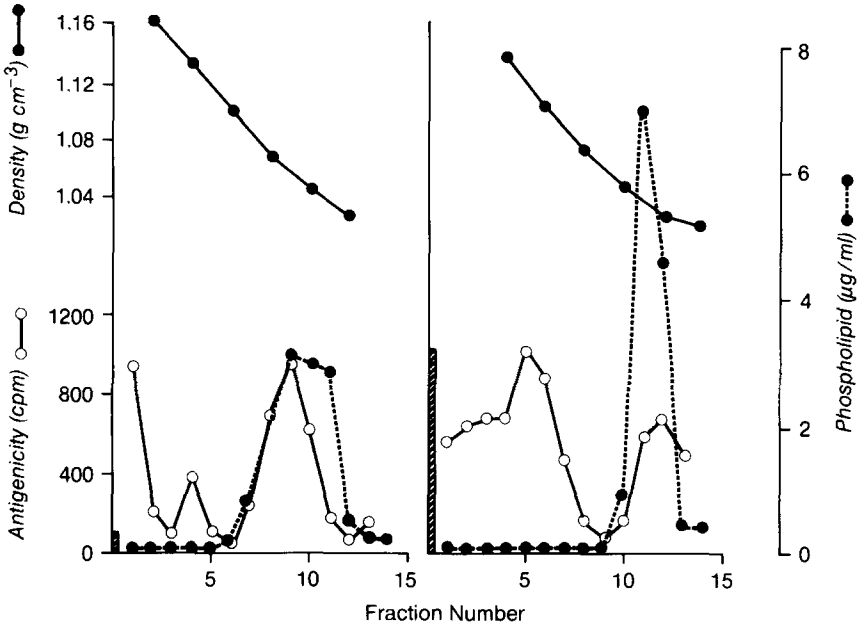


Figure 8. Most of the ECM SV4 antigen eluting from DEAE-Sephacel has the density of liposomes. The SV4 antigen from a DEAE column similar to that shown in Fig. 7 *A* was layered on a sucrose density gradient (5-60%) either before (*A*) or after (*B*) treatment with pronase. Fractions were assayed for phospholipid or antigenicity by dot blot assay on DEAE-nitrocellulose filters. Antigenicity in the pellets is shown by the hatched bar. Pronase-treated samples had barely detectable antigenicity when assayed by dot blotting on nitrocellulose filters. Most of the antigenicity had a density close to phospholipid before pronase (*A*) and the phospholipid peak was broad. Pronase digestion (*B*) separated most of the phospholipid from the antigenicity, some of which now pelleted. The hatched bar gives the antigenicity recovered in the pellet.

to that of the liposomes as detected by a lipid marker. It is likely therefore that these liposomes have the SV4 antigen inserted into the membrane with their antigen domain outside.

To determine if the pronase-resistant fraction is very close to the site of membrane association, an aliquot of the liposomes was treated with pronase before the centrifugation. If assayed on nitrocellulose paper, the antigenicity was essentially lost across the gradient (data not shown). If, however, the antigenicity in DEAE-binding material was measured, then the antigenicity could be recovered. Most was now found in the pellet and in high density fractions (Fig. 8 *b*). The liposome peak, denuded of antigen, had now a narrower density distribution. We conclude that pronase can shave the pronase-resistant antigenic domain from externally facing molecules.

The SV4 Antigen Is Associated with Nerve Terminals

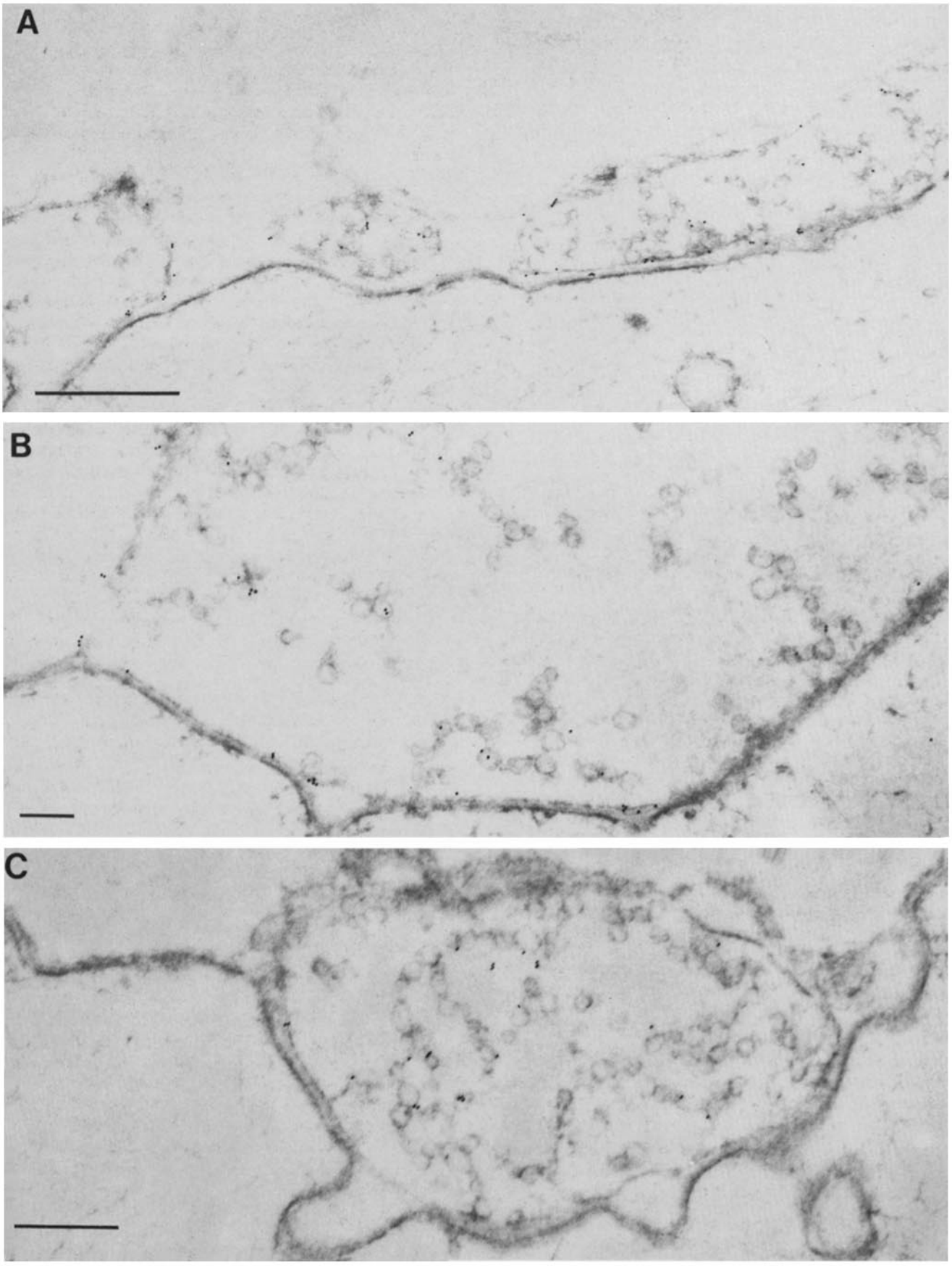
The biochemical evidence shows that the SV4 antigen in homogenates of electric organ is present in ECM as well as synaptic vesicles. Earlier immunofluorescence data (referred to in Caroni et al., 1985) showed the presence of the SV4 antigen on the outside of nerve terminals, in close association with the postsynaptic membrane. To measure how exclusively the SV4 antigen should be considered a nerve terminal marker in the electric organ, the subcellular location of the SV4 antigen was compared by immunoelectron microscopy with that of another synaptic vesicle antigen, the cytoplasmically located SV2. This 95-kD transmembrane glycoprotein (Buckley and Kelly, 1985) appears to be restricted to synaptic vesicles, and present in all synaptic vesicles. To allow accurate measurement, nerve terminals were sectioned, and antibody binding was assayed using gold bead-labeled second antibody.

Electric fish were perfused, their electric organs dissected, embedded with Lowicryl, and antibodies applied directly to the sections. The SV4 antigen (Fig. 9, *a* and *b*) is found associated with plasma membrane and vesicles while the SV2 antigen is primarily associated with vesicles (Fig. 9 *c*). The number of gold particles per terminal associated with the membrane and with the vesicles were measured for both antigens (Fig. 10 *a*). The data clearly show that SV4 is associated with the plasma membrane more than three times as much as SV2.

The SV4 antigen appears to be restricted to nerve terminal membranes. If the number of gold particles per length of nerve terminal membrane is compared to that for noninnervated postsynaptic membrane (Fig. 10 *b*) a clear preference for the nerve terminal membrane is seen. Preliminary measures of the distribution of the SV4 antigen along the nerve terminal plasma membrane have not yielded conclusive evidence for concentration in the synaptic junctional region in contrast to the results with horseradish peroxidase-labeled second antibodies (Buckley, K. M., unpublished observations; Kelly et al., 1986). The SV4 antigen is also, not surprisingly, absent from the highly involuted noninnervated face of the electrocyte. The number of gold beads contained in the cross-sectional area of this highly involved membrane was determined for eight micrographs. When this gold bead density was compared to that found over nerve terminals on the same micrographs, a 30-fold difference was seen.

We conclude that a portion of the SV4 antigen in electric organ is associated with the nerve terminal membrane. From the biochemical data, we are led to believe that a significant fraction of this plasma membrane antigen is ECM associated. These data also imply that the SV4 antigen associated with the nerve terminal plasma membrane is not due

Figure 9. Electron micrographic localization of the SV4 and SV2 antigens on the nerve terminals of electric organ. The appropriate monoclonal antibody was bound to ultrathin sections of electric organ embedded with Lowicryl K4M. Binding was detected using goat anti-mouse IgG coupled to 10-nm gold particles. (*A*) Three nerve terminals stained for the SV4 antigen. Bar, 1.2 μm. (*B*) A higher magnification of a nerve terminal stained for the SV4 antigen. Bar, 0.23 μm. Gold particles appear to be associated with both the synaptic vesicles and the periphery of the nerve terminal. (*C*) A nerve terminal stained for the synaptic vesicle antigen SV2. Bar, 0.58 μm.


A
B
C

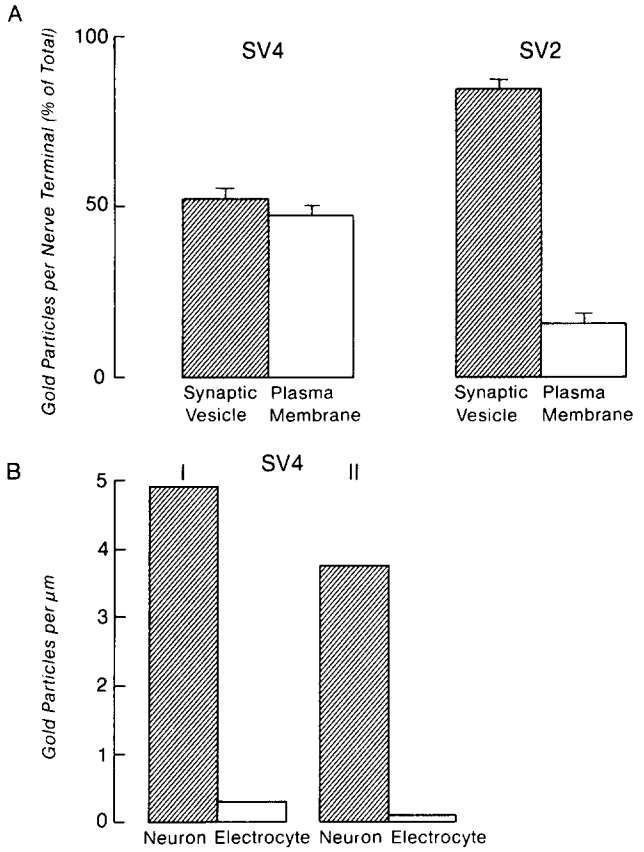


Figure 10. (*A*) The distribution of gold particles bound to the SV4 or SV2 antigen associated with synaptic vesicles or nerve terminal plasma membrane in electron micrographs. In 43 micrographs stained with a monoclonal antibody directed against SV4, the number of gold particles associated with either synaptic vesicles or nerve terminal plasma membrane was determined for 55 nerve terminals. For SV2, 24 micrographs were used and the gold particles bound to 29 nerve terminals were counted. The SV4 data represented two fish preparations, the SV2 only one. The data is presented in the bar graph as the average percentage of gold particles for each nerve terminal associated with either synaptic vesicles or nerve terminal plasma membrane. The error bars show the standard error of the mean for these averages. (*B*) The gold particle density (number/length of membrane) on nerve terminal plasma membranes (neuron) and non-innervated postsynaptic plasma membranes (electrocyte) from electron micrographs stained for the SV4 antigen. Lengths of plasma membrane which were well defined in the micrographs were measured and the number of gold particles associated with the measured region counted. The density was obtained by dividing the total number of particles counted by the total length of plasma membrane measured. For the SV4 antigen two separate fish were perfused, fixed, embedded, sectioned, and stained. The data from the two sets of electron micrographs are shown in the figure (labeled *I* and *II*). The total plasma membrane lengths measured were 54 (*I*) and 74 (*II*) μm while the electrocyte plasma membrane lengths were 9 (*I*) and 14 (*II*) μm.

to synaptic vesicle membrane awaiting recycling. Were this true, the SV4 and the SV2 antigens would have the same subcellular distribution, which is not found (Fig. 10).

Discussion

Since nerve terminals are often held in exact apposition to a specialized domain of postsynaptic membrane, some form of molecular bridge must hold the two membranes in register. Although the components of such a molecular bridge are unknown, electron microscopy, especially using quick-freeze techniques (Hirokawa and Heuser, 1982), clearly demonstrates physical links. Filamentous strands leaving the presynaptic membrane anchor it to the basal lamina, which is in turn anchored by similar strands to the postsynaptic membrane.

The distribution and characteristics of the protein described here make it a good candidate for a nerve terminal anchorage protein (TAP). By immunoelectron microscopy, it is present on the outside of the nerve terminal, and is absent from regions of the electrocyte that are not innervated. Presumably the molecule is transported down the axon by fast axonal transport (Caroni et al., 1985). It is firmly anchored in the basal lamina fraction from which it can be solubilized only by extreme denaturing conditions. Furthermore, the purified molecule binds to laminin affinity columns from which it can be eluted by heparin: the purified molecule also has a hydrophobic tail which allows it to incorporate into the bilayer of liposomes. It is an integral membrane protein of nerve terminals, as indicated by the presence of this hydrophobic domain, yet is attached firmly to the basal lamina. Thus, the molecule would seem by definition to fall into the class of anchorage proteins. With the caution that anchorage may not be the primary biological role we propose for the moment to describe the molecule as a terminal anchorage protein (TAP). Since more such molecules are likely to be discovered we refer to it as TAP-1.

There appear to be at least two ways that cells anchor to an extra-cellular substrate. The ECM proteins laminin and fibronectin bind either to conventional membrane protein receptors (Lesot et al., 1983; Rao et al., 1983; Terranova et al., 1983; Pytela et al., 1985; Malinoff and Wicha, 1983; Brown et al., 1983) or to glycosaminoglycan chains of heparan sulfate proteoglycans (Johansson and Höök, 1984) which may be membrane associated (for review, see Höök et al., 1984*a*). There is evidence that cells can anchor to their substrates by either mechanism but that complete rearrangement of the cytoskeleton requires both (for review see Lark et al., 1985). We have as yet no knowledge how much, if any, of the anchorage protein is exposed on the cytoplasmic face of the membrane.

The properties of the anchorage protein are quite unusual. Its size is very large. Depending on whether we compare its behavior on Sephacryl S-1000 in guanidine-HCl to proteins or to hyaluronic acid, estimates of size range from 10,000,000 to 350,000, respectively. It has at least one large pronase-resistant domain that can be separated from the membrane-associating region. This domain, eluting only at high salt from DEAE-cellulose, is presumably a region rich in negatively charged oligosaccharides. A cluster of glycosaminoglycan side chains has been proposed for a chondroitin sulfate proteoglycan with a protease-resistant domain (Höök et al., 1984*b*). A domain rich in serines and threonines in O-linkage with oligosaccharides has also been described for the low density lipoprotein receptor (Russell et al., 1984). Finally, the anchorage protein binds to laminin and can be eluted with heparin, implying a glycosaminoglycan-like domain. It is premature, however, to designate it a proteoglycan. Conventional enzymatic tests to verify the presence of glycosaminoglycan side chains have yielded ambiguous

results, even under conditions where standards were completely hydrolyzed. Perhaps the side chains are too closely packed. Alternatively, the molecule described here may resemble the large, negatively charged glycoprotein found in the growing tips of neuronal cells (Chernoff et al., 1983), although the latter elutes from DEAE at a much lower ionic strength. The unusual properties of the anchorage protein are not shared by the majority of the ECM proteins. Almost all of the other proteins were smaller in size on Sephacryl S-1000 and less negatively charged on DEAE-Sephacel.

Although the anchorage protein was first identified because it shared the SV4 antigen with the synaptic vesicle proteoglycan, they are clearly not identical molecules. Besides differences in size and charge density, they migrate differently on SDS polyacrylamide gels and the vesicle proteoglycan has an antigen (SV1) absent on the anchorage protein (Caroni et al., 1985). The two molecules may be related. For example, the vesicle may internalize a degradation product of the anchorage protein that no longer is firmly attached to the basal lamina. To establish a product-precursor or some other relationship between the two forms, information on the polypeptide backbones will be necessary.

The SV4 antigenic determinant is not on all ECM proteins, only the highly charged, large TAP described here, and the synaptic vesicle proteoglycan. It has been shown to have properties of an oligosaccharide and to be enriched in neurons innervating the electric organ (Caroni et al., 1985). Possible functions for this unusual determinant are targeting newly synthesized material to the correct intracellular destination or specifying the correct intercellular interaction between neurons and their electric organ target. Alternatively, the determinant could be involved in some function unique to electric organ.

The crucial role of the ECM in synaptic regeneration (Sanes et al., 1978) has energized the search for unique components of the synaptic junction. In addition to the usual components of the basal lamina, laminin, collagen, and fibronectin, the synaptic junction contains three unidentified antigens (Silberstein et al., 1982), a lectin (Sanes and Cheney, 1982), a clustering factor (Fallon et al., 1985), and a heparan sulfate proteoglycan that associates with acetylcholine receptor clusters (Bayne et al., 1984). It is striking that negatively charged proteoglycans are associated with receptor clusters in the postsynaptic membrane (Anderson and Fambrough, 1983; Bayne et al., 1984) and now with the presynaptic membrane that will bind to the clusters. Both proteoglycans could be bridging molecules anchoring their respective membranes to the basal lamina. If they are indeed components of a molecular bridge crossing the synapse, it will be important to discover if they form a universal synaptic glue or show synaptic specificity.

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