Isolation, Characterization, and Localization of the Spanning Protein from Skeletal Muscle Triads

Richard M. Kawamoto, J.-P. Brunschwig, Kyungsook C. Kim, and Anthony H. Caswell
Department of Pharmacology, University of Miami School of Medicine, Miami, Florida 33101

Abstract. A monoclonal antibody has been developed against the putative junctional protein or spanning protein (SP) from skeletal muscle triads. By immunoaffinity chromatography, we have purified this protein. The native protein has a molecular mass of 630–800 kD, as determined by gel filtration and rate zonal centrifugation. Within the limits of the methods used, the basic unit of the SP appears to be a dimer. In electron micrographs, it is shown to exhibit a circular profile with a diameter of ~100 Å. In thin section analysis, the protein is frequently observed as parallel tracks of electron-dense particles bordering a translucent core. We suggest that the basic unit of the junctional structure is a dimer of 300-kD subunits and that four such entities constitute the intact SP.

The purified protein has been used to develop polyclonal antibodies. By immunoelectron microscopy using immunogold probes, the SP has been localized to the junctional gap of the triad. By attaching the SP to an affinity resin, three proteins have been identified as forming associations with the SP. The M_rs of the proteins are 150, 62, and 38 kD; the 62-kD protein is calsequestrin.

The physiological mechanism of excitation–contraction coupling in skeletal muscle is not yet understood. It is currently believed that the signal transmission occurs at the triad junction where the transverse (T)-tubule[1] and sarcoplasmic reticulum (SR) membranes are closely apposed. Considerable morphological information is available regarding the triad junction, and it is clear that although the two membranes are separated by a gap of ~100 Å, the T-tubule and SR are physically joined and in communication by virtue of bridging structures that span the junctional space. These structures have been referred to variously as feet (13), bridges (21), pillars (11), and spanning protein (SP) (7). Biochemically, very little information is available about the junctional constituents. In an effort to understand the functional significance of the junctional complex, we have undertaken the task of elucidating the components that form the junctional architecture. The most likely candidates are proteins with molecular masses of 300, 270, 38, and 34 kD. The first two proteins are generally identified together as the high molecular mass doublet protein. They were identified as the putative SP that attaches to and joins the two organelles (4). The original evidence to support this proposal was the finding that the protein could jump from T-tubules to terminal cisternae SR when the junction was formed and broken. The assignation was further supported by the finding that proteases that break the junction hydrolyze this protein rapidly. In density gradient fractionation of microsomes, the SP has an identical distribution to calsequestrin, which is diagnostic of terminal cisternae SR. Its distribution also matches that of vesicles containing protrusions that may represent junctional feet. Proteins of M_r 38 and 34 kD were identified as junctional constituents (5). We have demonstrated that they are aldolase and glyceraldehydephosphate dehydrogenase, respectively (10). Glyceraldehydephosphate dehydrogenase catalyzes the formation of the triad junction from free T-tubules and terminal cisternae SR (9). The role of these glycolytic proteins in the junctional architecture is still uncertain. In this paper, we present the isolation and characterization of the high molecular mass SP.

1. *Abbreviations used in this paper*: Pristane, 2, 6, 10, 14-tetramethyl-pentadecane; SP, spanning protein; SR, sarcoplasmic reticulum; TC, terminal cisternae; T-tubule, transverse-tubule; UA, uranyl acetate.

Materials and Methods

Preparation of Monoclonal Antibody

Extraction and enrichment of SP was carried out as previously described (7). Briefly, vesicles of terminal cisternae (TC)/triads were treated with Triton X-100 (2 mg/mg protein). Membrane fragments containing components of the triad junction were solubilized by treating with a combination of NaCl and Zwittergent (3-14) detergent (CalBiochem-Behring Corp., La Jolla, CA). The solubilized samples were fractionated by molecular sieve chromatography with a Sephacryl S-400 column (1.5 cm diameter × 40 cm length, 0.6 ml/min flow rate). Fractions (1.8 ml) enriched with SP were identified by SDS gel electrophoresis and these fractions were used to immunize BALB/c mice. Mice were initially immunized (intraperitoneally) with 100 μg of protein mixed 1:1 with Freund's complete adjuvant (1 ml final vol). They were subsequently innoculated with 100 μg of protein mixed 1:1 with Freund's incomplete adjuvant (1 ml final vol) at 2 and 4 wk after the first injection. At 6 wk, the mice were challenged with 100 μg of protein mixed with PBS (1 ml) and sacrificed 3 d later. Fresh preparations of SP-enriched fractions were used for each injection. Development of monoclonal antibod-

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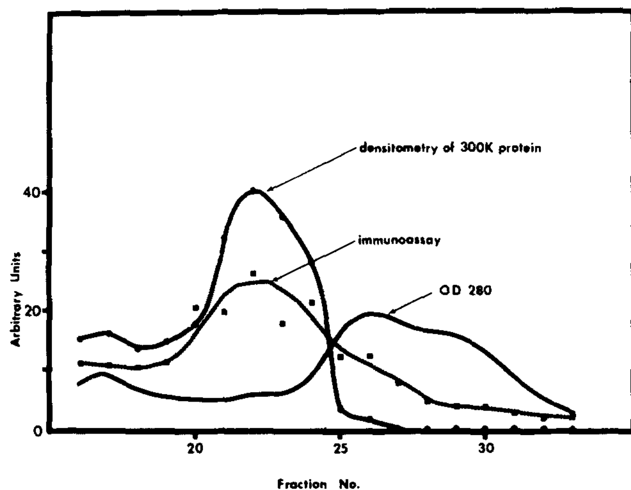


Figure 1. Identification of monoclonal antibody directed against the SP. The OD 280-nm trace is the protein elution from a Sephacryl S-400 chromatography of triad junctional proteins. The fractions were analyzed by SDS PAGE (80-μl samples/fraction) and the spanning protein was assayed by densitometry (●). Hybridoma culture media was reacted with column fractions and the antigen/antibody reaction (■) was determined by enzyme-linked immunosorbent assay procedures (see Materials and Methods). 300K protein, 300-kD protein.

ies was according to the method of Galfre and Milstein in which spleen cells were fused with SP/o myeloma cells (15). Dissociated spleen cells (60 × 10^6 cells) were mixed with myeloma cells at a ratio of 10:1 in a Dulbecco's modified Eagle's medium–hypoxanthine, aminopterin, thymidine selection medium. Fusion was accomplished with the addition of 50% polyethylene glycol. Hybridomas were initially screened against preparations of TC/triads by enzyme-linked immunosorbent assay procedures. Antigen was plated onto 96-well microtiter plates and incubated with hybridoma culture supernatant. Antigen/antibody reactions were detected with a peroxidase/antiperoxidase system (Sternberger-Meyer Immunocytochemicals, Inc., Jarrettsville, MD) using 4-chloronaphthol as substrate. During subsequent screenings the antigenicity of the SP was determined to be attenuated by procedures of Western blotting (3, 22). Consequently, alternative screening methods were necessary for the identification of hybridomas producing antibody against the SP (Fig. 1). Suspected hybridomas were cloned and incubated with fractions from the Sephacryl S-400 column output used for the preparation of enriched fractions of SP. The OD 280-nm trace shows the protein output of the Sephacryl S-400 column. Each fraction was resolved by SDS PAGE and the content of the SP was assayed by densitometry using a scanning densitometer (model SL-TRFF; Biomed Instruments, Inc., Fullerton, CA) (circles). The SP was found to match a small protein peak (fraction 22) that was distinct from the fractions where most of the proteins eluted (fractions beyond 24). Enzyme-linked immunosorbent assay procedures were performed on fractions of the S-400 column output that were dotted (5 μl) onto strips of nitrocellulose sheets. The strips were blocked with 3% BSA in PBS (2 h at 24°C) before incubating with culture media from hybridomas (overnight at 4°C). The strips were sequentially incubated with a biotinylated sheep anti-mouse IgG (1:300 dilution for 1 h at 24°C) and streptavidin/biotinylated peroxidase complex (1:400 dilution for 30 min at 24°C). Before reactions with the peroxidase substrate, 2,2′-azino-di-[3-ethyl-benzthiazolinsulfonate (6)] (Boehringer Mannheim Biochemicals, Indianapolis, IN), the individual dots on the nitrocellulose sheets were cut out and transferred to 96-well microtiter plates. The peroxidase/2,2′-azino-di-[3-ethyl-benzthiazolinsulfonate (6)] reactions were conducted in the wells and the extent of the reactions was quantitated by reading at 405 nm in a monitor (model EL 307; Bio-Tek Instruments, Inc., Burlington, VT) (squares). The peroxidase activity for a single clone was found to peak at fraction 22, coincident with the SP. A small shoulder was apparent at fraction 26 and it is probable this reaction indicates degradation products of the SP. The hybridoma producing antibody against SP was cloned twice by limiting dilution. Large quantities of monoclonal antibody were produced in mouse ascitic fluid. Mice were pretreated with 0.5 ml of Pristane (administered intraperitoneally). One million cells (in 0.5 ml of HT culture media) were injected into the mice (i.p.) 10 d after the Pristane pretreatment. Mice were tapped for ascites fluid ~9–10 d later. Ascites was centrifuged (clinical centrifuge, full speed) to pellet debris and the supernatant was dialyzed against 20 mM Tris-HCl (pH 7.4) (overnight at 4°C). Dialyzed ascites was then fractionated by column chromatography using a DEAE Affigel blue column (1.5-cm diameter × 40-cm length, Bio-Rad Laboratories, Richmond, CA) equilibrated with 20 mM Tris-HCl (pH 7.4). Elution was effected with a linear gradient of NaCl (0–150 mM) in 20 mM Tris-HCl (pH 7.4) (1.2 ml/min flow rate). Antibody fractions were stored in 20 mM Tris (pH 7.4), 30 mM NaCl, and 0.1% NaN_3 at 4°C until needed.

Immunoaffinity Column

Monoclonal antibody against the SP as obtained from the DEAE Affigel Blue chromatography was dialyzed against a buffer of 0.1 M $NaHCO_3$ (pH 8.3) and 0.5 M NaCl (coupling buffer) and coupled to CNBr-activated Sepharose 4B (Sigma Chemical Co.). The resin (1.5 g) was washed with 200 ml of 1 mM HCl by filtering the suspension through sintered glass attached to a vacuum pump. The gel was activated with 20 ml coupling buffer and filtered until nearly dry. The resin was removed from the sintered glass and mixed with the dialyzed monoclonal antibody solution (12–15 mg in ~10 ml) in a polypropylene culture tube (15 ml). The suspension was gently shaken for 2 h at 24°C, then filtered through sintered glass and quickly washed with coupling buffer. The resin was returned to the culture tube and mixed with 12 ml 0.2 M glycine (pH 8.3) to block unreacted groups on the Sepharose (shaking 2 h at 24°C). The resin was finally washed with alternating solutions of the coupling buffer and a buffer of 0.1 M $NaCH_3COOH$ (pH 4) and 0.5 M NaCl (four times). The washed column was equilibrated with PBS (pH 7.2), with 0.1% NaN_3 and stored at 4°C until use.

Isolation of the SP

TC/triads were prepared according to methods described (8) with minor changes. Both the homogenization and wash buffers contained 5 mM EGTA and 100 μM phenylmethylsulfonyl fluoride to minimize protease activity. The preparation was solubilized with 0.6 M NaCl and Zwittergent 3-14 (2 mg/mg protein). The solubilized sample (3 ml final volume) was gently shaken for 1 h at 30°C with the antibody coupled Sepharose 4B that had previously been equilibrated with 2 mM histidine (pH 7.0), 0.6 M NaCl, 1 mM EGTA, and 0.1% Zwittergent 3-14 (start buffer). After the incubation, the gel mixture was poured into a glass column and allowed to settle under flow. The settled column was initially washed with the start buffer to remove the large amount of unreacted vesicle proteins. This was followed by a solution of start buffer containing 4 M NaCl (~10 ml) to elute nonspecifically adsorbed proteins. Specifically bound proteins were then eluted with start buffer containing 4 M NaSCN in place of NaCl and collected in a fraction collector (1 ml/tube). The output from the column was monitored at 280 nm with a densitometer to detect the elution of the protein. Fractions from the SCN elution were analyzed by SDS PAGE. The NaSCN eluate containing protein was then pooled (~7 ml) and passed through a molecular sieve column (Sephacryl S-300, 1.5-cm diameter × 40-cm length) to separate the protein from detergent and salt. The column was eluted with 4 mM Tris (pH 7) and 30 mM NaCl (0.6 ml/min). The column output was monitored at 280 nm with a densitometer and collected in a fraction collector (1.2 ml/tube) for analysis by SDS-gel electrophoresis. As a control, an affinity column was constructed in a manner identical to the immunocolumn except no antibody was used.

Affinity-purified SP Antibodies

Polyclonal antibodies against the purified SP was raised in BALB/c mice following an immunization schedule similar to that described for the preparation monoclonal antibodies. Each innoculation contained 30–40 μg of purified SP either freshly prepared or previously stored frozen. The initial immunization was a 1:1 mixture with Freund's complete adjuvant and all subsequent injections have been with a mixture containing Freund's incomplete adjuvant. Blood obtained from the retro orbital venous plexus was allowed to stand at 24°C for 1 h in an Eppendorf microfuge tube. The clotted blood was centrifuged for 10 min in an Eppendorf microfuge to separate serum from the precipitated proteins. To affinity purify the SP polyclonal antibody the serum was collected and incubated with an SP-coupled Sepharose 4B resin (0.5 ml, see below) for 1 h at 24°C with gentle shaking. The mixture was then poured into a glass column and the output (gravity flow) was monitored at 280 nm with a densitometer. The column was first washed with 0.3 M NaCl, 4 mM Tris-HCl (pH 7.0) and 0.1% NaN_3. The second wash was with the same solution except that the NaCl was increased to 1 M. Poly-

clonal antibody was specifically eluted with the same solution containing 4 M NaSCN instead of NaCl. The NaSCN eluate was collected (~2 ml) and dialyzed against 20 mM Tris-HCl (pH 7.4) and 0.1% NaN_3 and stored refrigerated.

SP Affinity Column

SP was prepared by passage of dissolved TC/triads through the antibody column as described above with the exception of substituting 2 mM sodium phosphate (pH 7.0) in place of the histidine in the eluting buffer for the antibody column. The NaSCN eluate was coupled directly to CNBr-activated Sepharose in a manner similar to procedures used for the construction of the immunoaffinity column. The pH of the SCN output was adjusted to 8.3 by titrating with NaOH before the reaction of the SP and the CNBr-activated Sepharose. A control column was similarly constructed by treating CNBr-activated Sepharose with the NaSCN eluting buffer adjusted to pH 8.3. The columns were equilibrated with 2 mM histidine (pH 7.0), 30 mM K gluconate, 1 mM EGTA, and 0.1% Zwittergent (wash buffer).

TC/triads (1–2 ml, ~20 mg/ml) were resuspended in 2 mM histidine (pH 7.0), 250 mM sucrose, 1 mM EGTA, and 100 μM phenylmethylsulfonyl fluoride. The vesicles were treated with 0.3 M K gluconate and Zwittergent (2 mg/mg protein). The suspension was diluted 10-fold with water to reduce the K gluconate concentration. The mixture was centrifuged (Airfuge Ultracentrifuge; Beckman Instruments Inc., Fullerton, CA) for 30 min at 30 psi. The supernatant was passed through the SP affinity column. The column was washed with the wash buffer until the densitometer trace had returned to baseline and specific elution was achieved with a solution of 2 mM histidine (pH 7), 1 M NaCl, 1 mM EGTA, and 0.1% Zwittergent.

Rate Zonal Centrifugation

Samples (250 μl) were layered on top of a continuous linear sucrose gradient of 5–30% sucrose (wt/wt) containing 30 mM NaCl. The samples were centrifuged in a Beckman SW 56 Ti rotor for 16 h at 29,000 rpm. Fractions (250-μl) were collected from the bottom of the tube and analyzed for protein colorimetrically (150 μl/fraction, reference 1) or by SDS PAGE (80 μl/fraction). Densitometry of the Coomassie Blue–stained gels was performed for analysis of the SP.

Negative Stain

SP obtained from the S-300 chromatography was concentrated by centrifugation using a centricon filter (Amicon Corp., Danvers, MA) and stained for electron microscopy as described previously (17). Briefly, 5-μl aliquots of the protein were spotted onto a small plug of 2% agar made in physiological saline (1-cm diameter and 2-mm thick). Each spot was allowed to dry before subsequent applications; as many as five drops were used. The agar surface was coated with a film of 0.75% parlodion in amyl acetate. Once dry, the agar plug carried on the edge of a glass microscope slide was dipped slowly at an angle into a pool of 0.2% uranyl acetate (UA). The parlodion was peeled off the agar and floated on the surface of the UA bath. A 400-mesh copper grid was placed on the film in the area spotted with protein. A brass rod with a diameter slightly larger than the grid was placed against the grid and immersed into the UA solution. This caused the film of parlodion to wrap around the rod securing the grid to the flat surface of the brass rod. The grid was lifted out of the UA bath and gently blotted to leave a fine film of stain on the protein/grid surface and left to air dry. Electron microscopy was performed with a Phillips 300 or a JEOL-100 CXII electron microscope operating at 80 kV. Similarly, ferritin was treated as described and used as a control for sizing. In this and subsequent treatments of the purified SP, the dimensions of the protein (mean ± SD) were determined on 50 measurements by three individuals and averaged.

Thin Section

The output from the Sephacryl S-300 column containing the SP was pooled and pelleted by centrifugation using a Beckman SW 41 Ti rotor (32,000 rpm for 16 h). The pellet was fixed with glutaraldehyde and then with OsO_4 as described before (2). The sections were poststained with UA and counter stained with lead citrate. Ferritin was similarly pelleted, fixed, sectioned, and poststained for thin section microscopy.

Rotary Shadowing

Samples were prepared according to the method previously described (12) with minor alterations. A 5-μl drop of the concentrated SP was placed on a support of freshly cleaved mica. As a control, ferritin was placed on a similar piece of mica and treated simultaneously with the SP sample. The pieces of mica were washed gently with a solution of 2% UA and then deionized water. The samples (SP and ferritin) were mounted together on a Balzers (Hudson, NH) specimen holder, covered with an aluminum foil cap, and quickly placed into liquid nitrogen. The frozen samples were rapidly inserted and mounted onto the stage of a Balzers 300 freeze-fracture chamber precooled to −150°C. The temperature was raised to −105°C and the specimen cap dislodged by bumping it with the Balzers knife holder arm. Freeze drying of the samples was carried out for 30 min. The protein was rotary shadowed by holding the Pt/C electrode at 10° for 30 s while rotating the stage at 1 rps. Carbon was then deposited at 90° for 10 s. Replicas were lifted off in water and placed in bleach to remove biological material. Negatives obtained from the microscopy were reversed before printing.

Immunogold Localization

Small strips (1 mm × 3–4 mm) of rabbit sacrospinalis muscle were excised and lightly fixed with 0.5% glutaraldehyde and 3% paraformaldehyde in PBS (pH 7.3) for 1–1.5 h. The tissues were rinsed (three times) in 0.1 M cacodylate buffer (pH 7.2) for 1 h and dehydrated in a series of ethanol steps. All steps were carried out at 4°C. The tissue was placed in precooled gelatin capsules, oriented and immediately embedded with "LR white" hard grade resin (The London Resin Co., Ltd., Schenectady, NY), polymerized with accelerator for "LR white" (Polysciences, Inc., Warrington, PA) 1 drop/10 ml of resin at 0°C and stored frozen until ready to section. Fresh thin sections were cut with a diamond knife and placed on 400-mesh nickel grids. Grids were floated, section down, on drops (10–20 μl) of reagents placed on a sheet of parafilm at room temperature. They were washed on larger drops of PBS and distilled water. All reagents except the immunogold and the polyclonal antibodies were passed through 0.22-μm millipore filters (Millipore Corporation, Bedford, MA) immediately before use.

Grids were first incubated for 1 h in 0.5% BSA, 0.1 M glycine, 250 mM sucrose, and 50 mM Tris acetate (pH 7.2). They were then placed in undiluted polyclonal antibody against the SP for 30 min except the control. Next the grids were washed by passing over 12 drops of 250 mM sucrose, 50 mM Tris acetate (pH 7.2) (Tris-sucrose buffer) and left for 15 min on the last two drops. This was followed by incubation on a drop of goat antimouse IgG adsorbed to 5-nm colloidal gold particles (AuroProbe EM GAMIgG G5, Janssen Pharmaceutica, Piscataway, NJ) for 20 min. The grids were washed with Tris-sucrose buffer as before, left for 10 min in the last drop, and finally passed through nine drops of distilled water. Incubation on a drop of 3% glutaraldehyde in PBS (pH 7.2) for 5 min was followed with Tris-sucrose buffer and distilled water as in the step above. Before observation under the electron microscope, specimens were stained for 5 min with UA and counter-stained for 3 min with lead citrate.

Amino Acid Analysis

Purified SP was pelleted (Beckman SW 41 Ti rotor, 16 h at 32,000 rpm) and resuspended in 100 μl PBS. The sample was hydrolyzed with 6N HCl for 22 h at 110°C in vacuo. Amino acid analysis was carried out with a JEOL 5AH amino acid analyzer using single column methodology ninhydrin detection.

Gel Electrophoresis

SDS PAGE was run according to methods previously described (16) using 5% acrylamide and 0.13% N,N'-methylene-bis-acrylamide. Samples were boiled for 1 min and immediately loaded onto the stacking gel consisting of 3% acrylamide and 0.08% bis-acrylamide. Gels were either stained with Coomassie Blue or silver (18).

For Stains-All staining, the gels were exhaustively destained with 25% isopropanol with solid Tris added to alkalinize the gel. The gels were subsequently stained with Stains-All (6).

Protein Standards

Phosphorylase kinase from rabbit was a gift from Dr. Ernest Lee, ovostatin was a gift from Dr. J. F. Woessner, and heavy meromyosin was a gift of Dr. P. Strang (all from University of Miami School of Medicine). Rabbit phosphorylase a, bovine thyroglobulin and horse ferritin were from Sigma Chemical Co. Glyceraldehydephosphate dehydrogenase was prepared from rabbit skeletal muscle as previously described (9).

Results

Isolation

Fig. 2 illustrates the extraction of the SP from TC/triads by the antibody column. Lane *1* represents the preparation of TC/triads used for the extraction of the SP. Lane *2* is the NaSCN output from the antibody column and lane *3* is the same fraction from a control column. From lane *2*, the specific nature of the monoclonal antibody is observed. With a single step, the high molecular mass doublet and a small amount of a protein of M_r 140 kD (suspected to be a proteolytic fragment of the SP) are extracted from a milieu of triadic proteins. By comparison, the control column is devoid of any retained proteins.

Fig. 3 shows the profile of the eluate from the immunoaffinity column after fractionation by molecular sieve chromatography on a Sephacryl S-300 molecular sieve column. Three peaks are detected in the OD 280-nm trace. A large initial peak occurred at tube 26, a shoulder at ~33 and a final peak at tube 35. The last peak is largely a light-scattering artifact attributed to the detergent elution. The SP eluted as two distinct fractions. Most of the high molecular mass SP eluted in the first peak. From column calibrations, the material eluted has a native molecular mass of ~630 kD. The SDS-gel profile indicates that a protein with M_r of nearly 300,000 is the principal and almost sole component contained in the first peak (tube 26). The second fraction at tube 33 is largely composed of the 300-kD protein. In addition, a minor protein of M_r 140,000 is present in this fraction (arrowheads). It is likely that the first peak represents protein that has repolymerized into a dimer after reduction of salt and detergent concentrations in the column while the second peak may be of monomeric protein released from the affinity column by the NaSCN. In our more recent experiments in which both EGTA and phenylmethylsulfonyl fluoride have been included to reduce protease activity, we have found that the higher M_r component of the doublet is the predominant protein of intact triads and of the isolated SP. This suggests that the 270- and 140-kD subfractions may represent proteolytic fragments of the parent 300-kD protein.

Fig. 4 shows the results of a rate zonal centrifugation analysis. The purified SP (first peak from the molecular sieve chromatography) distributed broadly (squares) with a peak at fraction 11. From the molecular mass standard curve (circles), the peak at fraction 11 represents a native molecular mass of ~800 kD (short arrow). The SP distribution also indicates a small amount of a lesser molecular mass species, 100–300 kD, which is likely to be a monomeric form as well as some higher M_r material, which presumably represents higher polymeric or aggregated forms.

TC/triads (5 mg) dissolved in Zwittergent and NaCl were similarly analyzed by rate zonal centrifugation (triangles). The SP obtained in this manner shows a predominance of a peak at fraction 8 along with a significant peak at fraction 11. The peak at fraction 8 corresponds to a molecular mass of 450 kD (long arrow). This suggests that the dissolved TC/triads yield both monomeric and dimeric forms of the SP. The detergent is still present in the preparation and may contribute to the behavior of the protein. In the case of the purified protein, the apparent lack of this smaller species is not unexpected, as the fraction used from the molecular sieve selected against the monomeric form of the protein. Our data are consistent with the view that a monomeric form of the protein is formed on dissolution of the TC/triads while a polymer (probably a dimer) is regenerated when the detergent and salt are diminished by the Sephacryl column. However, the rate zonal centrifugation, the molecular sieve chromatography, and SDS gel electrophoresis do not give identical M_r estimates. It must be accepted that some error in all the estimates will occur when the apparent M_r of the protein is so high.

Table I gives the analysis of amino acids. Methionine and

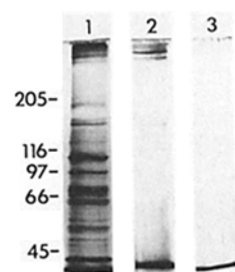


Figure 2. Antibody-affinity purification of SP from TC/triads. TC/triads (7 mg) were dissolved with Zwittergent 3-14 (2 mg/mg protein) and 0.6 M NaCl (3 ml final volume). The sample was incubated with the antibody affinity resin (preparation in Material and Methods) for 1 h at 30°C while gently shaking. Specifically bound protein was eluted with a solution containing 4 M NaSCN. Lane *1*, preparation of TC/triads used for the extraction of the SP (10 µg). Lane *2*, NaSCN eluate from the affinity column (25 µl). Lane *3*, NaSCN eluate from a control column without coupled antibody (25 µl). The gels were developed by silver staining.

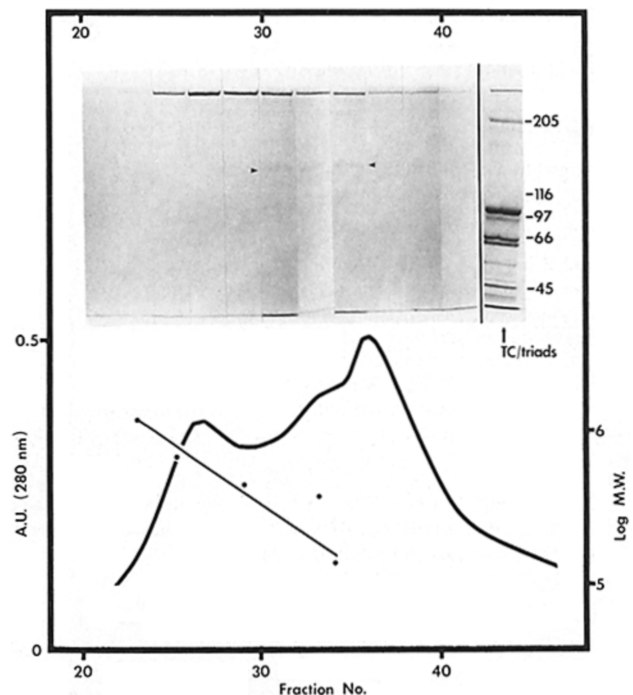


Figure 3. Gel filtration of the affinity-purified 300-kD protein. The output from the affinity column was fractionated by Sephacryl S-300 chromatography (see Materials and Methods). The column output was monitored at 280 nm and each fraction was resolved by gel electrophoresis (150 µl/fraction and 24 µg of TC/triads was used for the gel analysis). The gel was stained with Coomassie Blue. Arrowheads, the 140-kD protein. The molecular mass standards are expressed as $\times 10^{-3}$. The Sephacryl column was calibrated with the following standards: phosphorylase kinase (1.2 kD), thyroglobulin (670 kD), ferritin (450 kD), phosphorylase a (380 kD), and glyceraldehydephosphate dehydrogenase (140 kD).

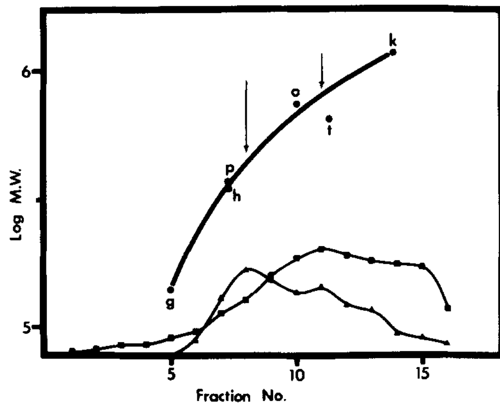


Figure 4. Molecular mass determination by rate zonal centrifugation. 250-μl samples of protein standards (●), purified SP (■) and SP from dissolved TC/triad proteins (▲) were layered on a continuous linear sucrose gradient. Fractions were analyzed for protein as described in Material and Methods. The curves for purified SP and TC/triads are densitometry scans for SP after resolving by SDS PAGE. Short arrow, ~800 kD; long arrow, ~450 kD. Protein standards were: k, phosphorylase kinase (1.2 kD); o, ovostatin (800 kD); t, thyroglobulin (670 kD); p, phosphorylase a (380 kD); h, heavy meromyosin (350 kD); and g, glyceraldehydephosphate dehydrogenase (140 kD).

Table I. Amino Acid Composition

Amino Acid	Mol/100 mol amino acid
Aspartic acid	10.05
Threonine	4.50
Serine	6.98
Glutamic acid	14.36
Proline	4.68
Glycine	14.20
Alanine	7.61
Cysteine	–
Valine	5.56
Methionine	–
Isoleucine	3.52
Leucine	10.14
Tyrosine	1.01
Phenylalanine	3.81
Histidine	3.13
Tryptophan	–
Lysine	4.11
Arginine	6.34

tryptophan are not resolvable by the protocol. Cystine should have been discernible but cysteine identification is difficult. Based on this observation it would be difficult to predict the existence of any disulfide bridges. The protein is neither strongly hydrophobic nor polar.

Electron Microscopy

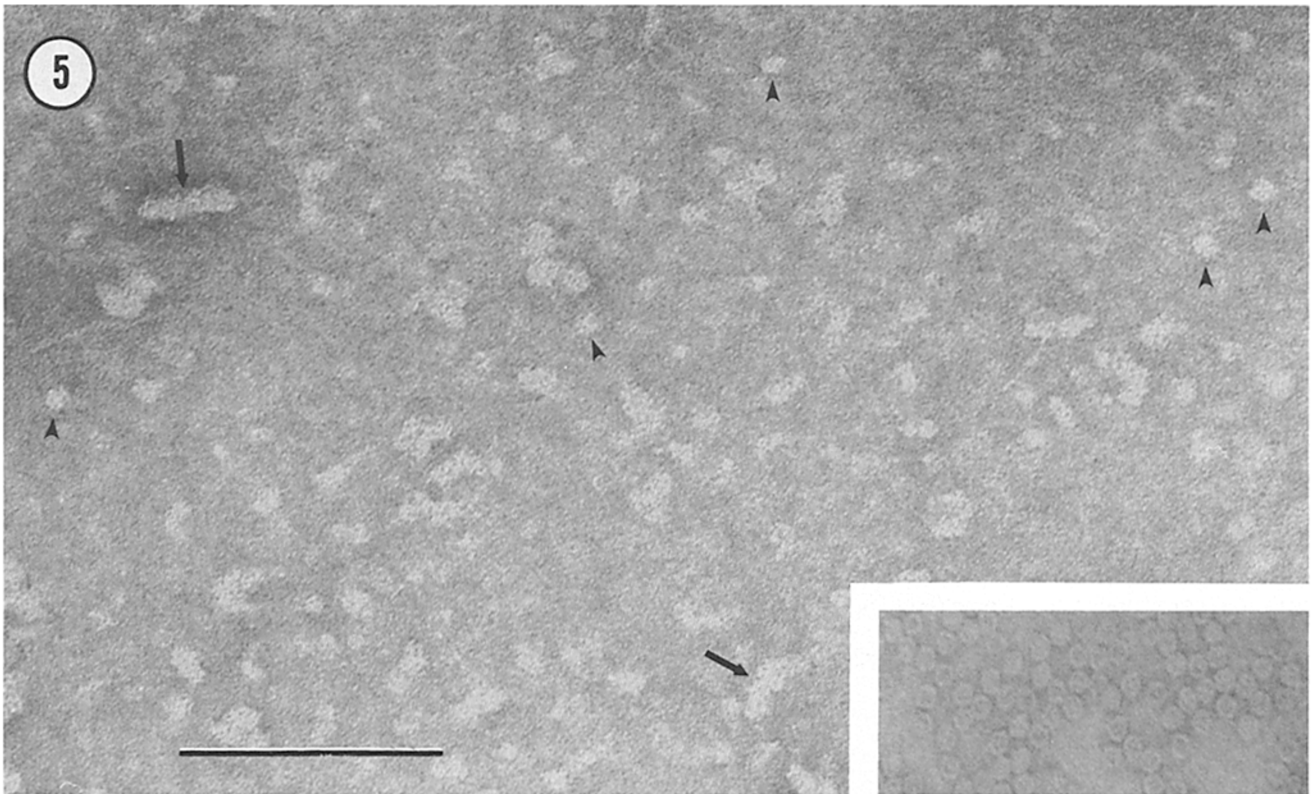
Fig. 5 is a view of the purified protein as seen by negative stain electron microscopy. The protein appears to be globular and in many instances an approximately circular profile is evident (arrowhead). However, the boundary is frequently uneven and the appearance is presumably dependent on the orientation of the protein. These structures have a diameter of ~93 ± 14 Å. Frequent clusters of the SP (arrow) are visible in the field showing a tendency by the protein to associate. These clusters are sometimes approximately linear and sometimes disorganized. For comparison, negative stain images of ferritin particles are shown in the inset. The ferritin molecules are clearly circular with a diameter of ~85 ± 4 Å.

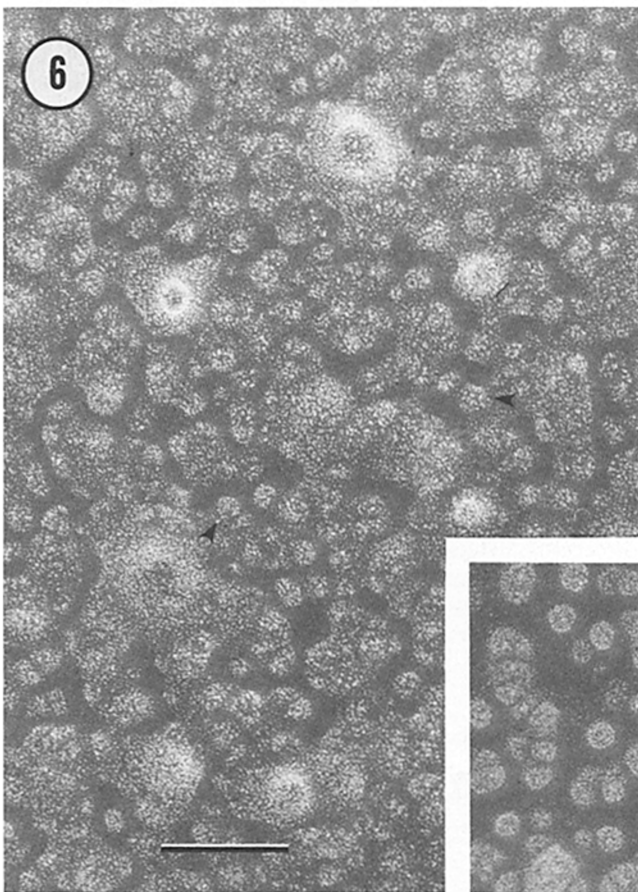
The circular appearance of the SP is substantiated by the images obtained by rotary shadowing (Fig. 6). A number of particles are seen with approximately circular profile and diameter of ~171 ± 22 Å (arrowhead). The increase in dimension of the small particles compared with negative stain is attributable to the accretion of platinum, which occurs using a low angle shadowing procedure. In addition, indistinct clumps or aggregates are seen. This tendency to aggregate appears as a characteristic feature of the molecule and complicates analysis. Extensive aggregation was not observed with ferritin. Again, rotary-shadowed ferritin molecules are provided for comparison (inset). Ferritin in these images have a diameter of ~185 ± 19 Å. The increase in the diameter of the ferritin molecules compared with the negative stain images is then ~100 Å. If this value is subtracted from the measured diameter of the SP, the SP diameter is ~71 Å, which, in view of the inherent errors of the protocols, is in fair agreement with the estimate in negative stained images.

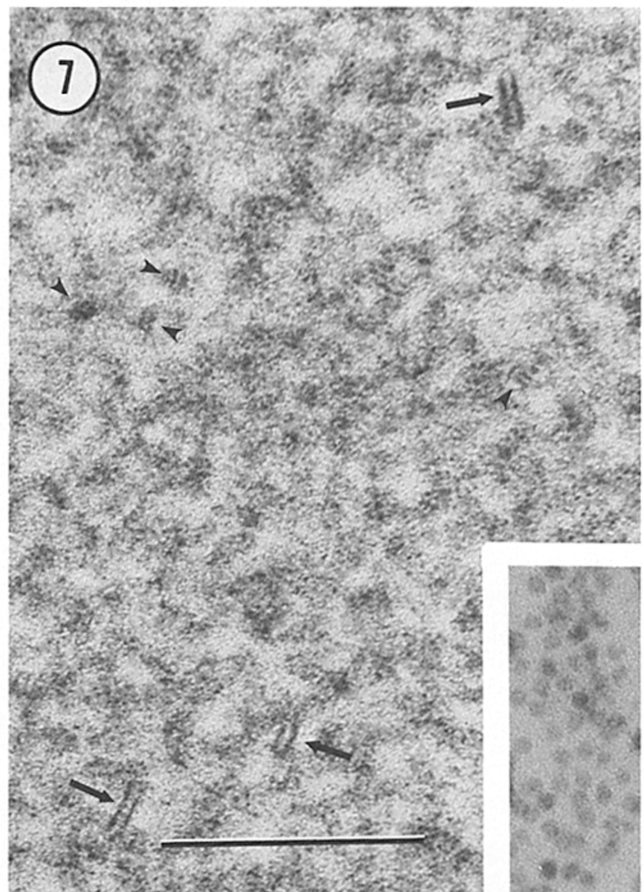
Fig. 7 shows thin section views of the precipitated SP. The amorphous appearance of the field is due to pelleting a large amount of material and the thickness of the section relative to the actual size of the protein units. A representative field contains frequent small particles that exhibit an electron dense core. The most striking features within the field is the presence of parallel tracks of electron dense structures (arrow). These images vary in length but the short dimension across the tracks is ~65 ± 6Å. For a comparison, in the thin section images of ferritin (inset), the protein diameter is ~67 ± 6 Å. The ferritin has been treated to stain the entire molecule so that what is seen is not just the iron core, which has been reported to have a diameter of 50 Å (19). The smaller diameter of ferritin compared with negative stained images is likely to be caused by shrinkage during the sample preparation and is therefore, not inconsistent with the diameter described by thin sectioning. What is not so obvious but more prevalent is the occurrence of much shorter images of parallel electron dense bodies (arrowhead). The smaller tracks may represent sections through individual 93-Å diameter molecules and the degree to which these tracks are discernible reflects the plane of section through the protein. The less frequent but more striking elongated tracks may then represent ideal longitudinal sections through the linear aggregates observed by negative staining.

Localization

Fig. 8 (*a–d*) demonstrates the immunolocalization of the SP in intact rabbit sacrospinalis muscle using polyclonal antibody against this junctional protein. The tissue was embedded in LR white under cold conditions to minimize loss of antigenicity. The immunolocalization reactions were performed following the embedding and sectioning of the samples. Fig. 8 *a* is a control section in which the primary antibody has been omitted, showing a field of sarcomeres in which the triadic regions are distinguishable. In most in-







Figures 5–7. (Fig. 5) Negative stain images of the purified SP after Sephacryl chromatography. *Arrowhead*, individual units. *Arrow*, polymeric forms. The insets in Figs. 5–7 show a fields of ferritin molecules as control. (Fig. 6) Isolated SP as resolved by rotary shadowing. *Arrowhead*, individual units. (Fig. 7) Thin section of the isolated SP. *Arrow*, long parallel tracks of electron-dense material. *Arrowhead*, short segments of parallel tracks. Bars, 100 nm.

stances, the triad identification is facilitated by the electron opaque clumps localizing calsequestrin. Although the contractile proteins are well preserved, the vesicles have swollen and the membranes are only distinguishable in some regions. The SP antibody was not used and a few gold particles are scattered randomly through the field. By comparison, in sections incubated with the SP antibody, gold particles are clearly visible and are localized in regions outlined by membranes of the T-tubule and terminal cisternae SR (Fig. 8 *b*). Scattered particles presumably associated with nonspecific antibody binding is seen in other regions, but the distribution is random and the density is quite low. By comparison, the control sample (Fig. 8 *a*) in which SP antibody was not used is almost devoid of gold particles despite the retention of morphology (Fig. 8 *b*). Fig. 8, *c* and *d* are views of the triad junction in which the section is along the T-tubule. A clear arrangement of the gold particles is seen to align along the membrane. The gold particles are primarily localized in the junctional gap and are generally absent from regions of calsequestrin or T-tubule lumen except in the immediate vicinity of the junction. Occasionally, gold is seen in the T-tubule lumen that may be nonspecific absorption. However, an ambiguity is present of ~10 nm. This ambiguity is attributable to the double antibody-labeling procedure as well as to the fact that the sample is viewed through the full thickness of the sections, whereas the antigenicity is confined to the surface.

Interaction of the SP with Triad Junctional Proteins

The interaction of SP with other triad proteins was investigated by forming an affinity column of SP with Sepharose and passing through a preparation of dissolved TC/triads (Fig. 9). It was necessary to dissolve the vesicles in a medium containing moderate salt concentration since prior experience has shown that the junction does not dissolve in the absence of salt, whereas high salt concentrations dissociate the proteins fully. The proteins were passed through the column in 30 mM K gluconate and then bound proteins were eluted with 1 M NaCl and concentrated using a Centricon membrane before gel analysis. The SP column specifically retarded proteins with M_r of 38, 62, and 150 kD (lane *3*). The control column retarded only a small amount of the 38- and 62-kD proteins with a noticeable lack of the 150-kD protein (lane *5*). When the gel was stained with Stains-All (lanes *2*, *4*, and *6*), the bands at 62 and 150 kD stained blue. The 62-kD protein can by this means and by silver staining in which it is colorless be identified as calsequestrin. The 150-kD protein is one of the two previously described (6). The M_r of the 38-kD protein suggests that it is aldolase because we have previously demonstrated that aldolase is an extrinsic constituent of isolated terminal cisternae SR and is the major band at 38 kD. The three proteins that are retarded by the affinity column are all SR proteins rather than T-tubular constituents.

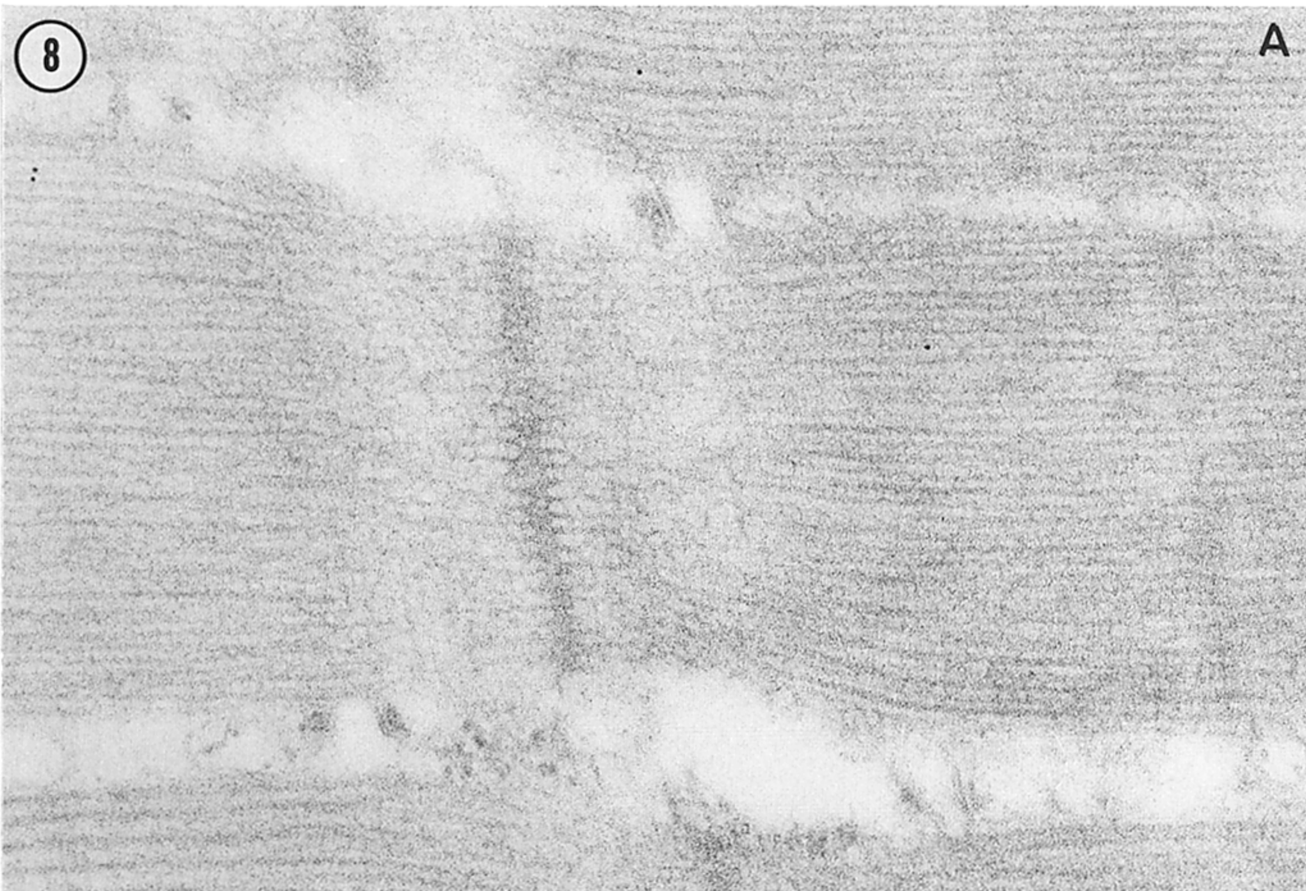
Discussion

The development of a monoclonal antibody against the putative SP of the triad junction of skeletal muscle has enabled us to obtain an essentially pure preparation of the protein in a single step. The subsequent protocol of gel filtration chromatography has been used to diminish the salt and detergent which were introduced to dissolve the triads. The antibody column can be used several times but deteriorates over a period in excess of a month; it can readily be replaced since the supply of antibody is unlimited. The very low contamination with lower M_r constituents probably represents breakdown products of the parent protein since essentially no contamination of polypeptides corresponding to the major SR bands is discernible.

In an earlier publication, on the basis of molecular sieve chromatography (7), we estimated the native M_r of the SP to be 1.2 M. We have reevaluated the M_r of the native purified protein to determine whether the isolation protocol has caused any alteration in the properties of the molecule and to provide a more reliable M_r estimate than that of chromatography, which is known to be susceptible to protein binding and hydrophobic repulsion. It appears that after dissolution, the protein is partially monomeric and partially dimeric, and a small amount of higher polymerization occurs. After the protein has been purified and fractionated by chromatography, the predominant form is apparently dimeric with higher aggregates present. We consider the dimer of the protein to be the most likely physiological form because it is produced when artificial conditions of high salt and detergent are diminished.

The electron micrographs of the first peak of SP from the molecular sieve column confirms the conclusions above that the protein has a tendency to aggregate. This aggregation may be accentuated in the preliminary treatment of the sample for electron microscopy. The propensity for aggregation complicates the interpretation of the micrographs. The negative stain provides the most accurate indication of the surface outline of the protein. It indicates an approximately spherical but irregular surface. The thin section reveals internal structure of the protein with an electron translucent core. This core could be aqueous, lipid, or be composed of hydrophobic (and therefore, nonreactive to stain) amino acid residues. A comparison of our isolated protein with electron microscopic evidence of the junctional "foot" reveals considerable consistency. These junctional structures have recently been described as quatrefoils (12). The 71 Å units described here could represent a single unit of the quatrefoil if account is taken of the contribution of the platinum deposit in enhancing the apparent size of the unit in shadow casting. The junctional elements have been described as having a diamond pattern in thin sections of intact muscle (14) and similarly, they have been seen as 200 Å diamond patterns in negative stain of isolated triads (20). This could represent the complete quatrefoil described (12) and therefore be composed of four units of 100 Å each. The diamond pattern images (14,20) as well as our own thin section images suggest that 100 Å units may be observed. The bridges have also been described to have an electron-translucent core in thin section of intact muscle (21). This conforms with our observation in thin section that the SP is electron translucent in the center.

Our original identification of the 300 kD dimer of the SDS gel pattern as the spanning of the triad was based on the observation that this protein could be transferred from T-tubules to terminal cisternae SR if the triad was reformed and then broken (7). This identification was indirect although quite specific. Further evidence to support this analysis included the finding that it was hydrolyzed by proteases that also broke the junction. Moreover, we observed that it matched the distribution of calsequestrin not only in continu-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A

B

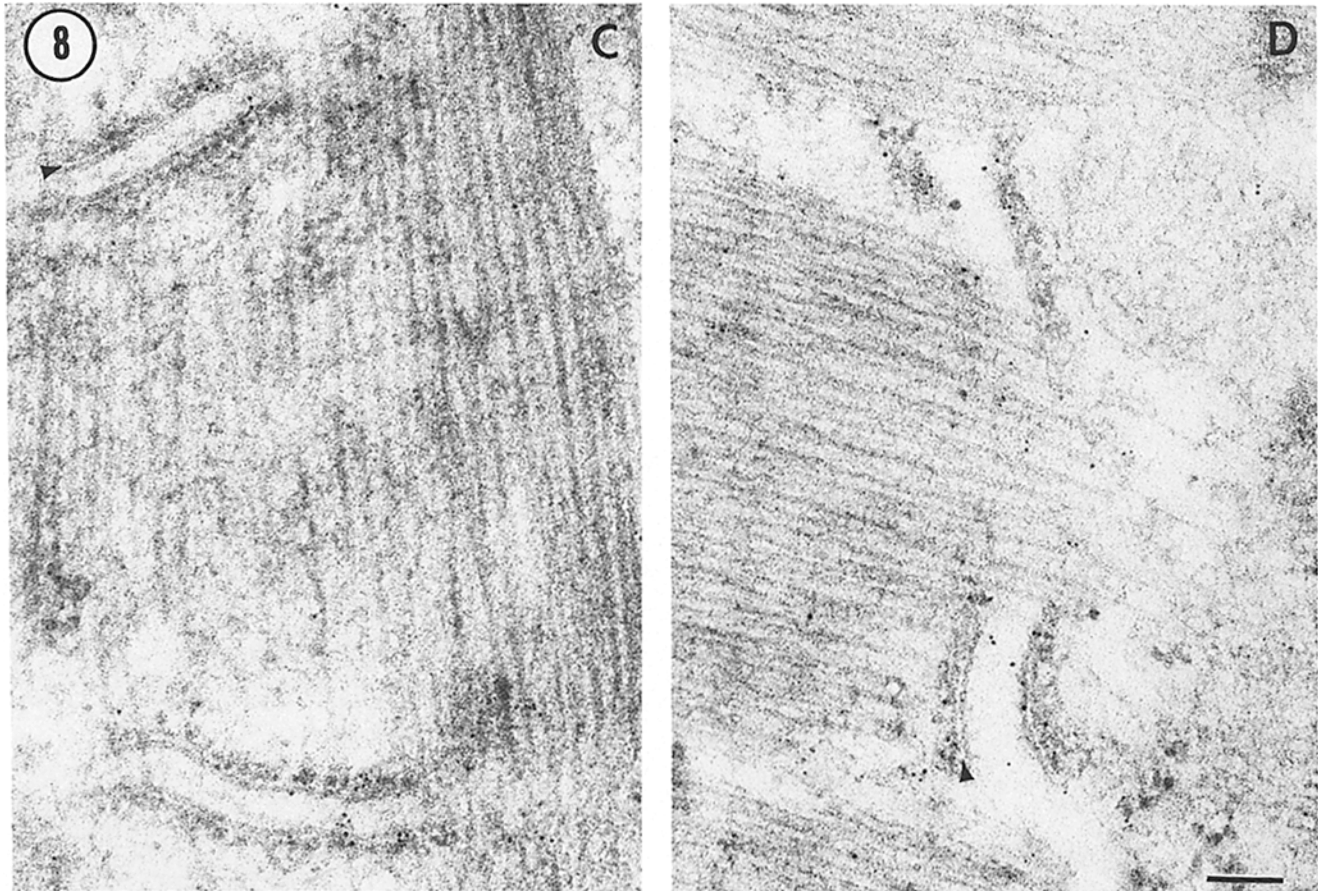

Figure 8. Immunogold localization of the SP in intact skeletal muscle. (*A*) Control section of muscle treated similar to (*B*) but in which the polyclonal antibody incubation was omitted. (*B*) Thin sections of muscle were reacted with polyclonal antibody against the purified SP and detected with gold-labeled sheep anti-mouse IgG. (*C* and *D*) Thin sections of muscle in the plane of the T-tubule. Both sections have been incubated with the polyclonal antibody and visualized by immunogold labeling. *Arrowheads*, the triad junctional gaps stained with colloidal gold.

ous sucrose gradients of microsomes and broken triads, but also in being retained with calsequestrin in junctional fragments after Triton treatment (4).

In this paper we provide further and extensive corroboration of our initial identification. (*a*) As stated above, our electron micrographs conform with those of junctional feet in intact muscle and isolated triads or terminal cisternae. (*b*) We

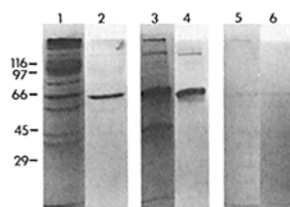

Figure 9. Interaction of the SP with junctional proteins. Purified SP was coupled to CNBr-activated Sepharose. Proteins from dissolved TC/triads were reacted with the immobilized ligand and specifically associated proteins were eluted with a solution containing 1 M NaCl (see Materials and Methods). Eluted fractions (2 ml) were concentrated (70 μl) with a centricon filter and all of the samples were resolved by SDS PAGE and detected with Coomassie Blue (odd numbered lanes). The same gels were destained and subsequently stained with Stains-All (even numbered lanes). (Lanes *1* and *2*) Preparation of TC/triads used in the reaction (15 μg). (Lanes *3* and *4*) NaCl eluate from the SP affinity column. (Lanes *5* and *6*) NaCl eluate from a control column in which the SP was omitted from the column preparation protocol.

also show by immunoelectron microscopy that antibodies against the SP are found in the junctional region. If we exclude some nonspecific gold particles distributed evenly in the section, the particles are predominantly localized in the region at which the T-tubule membrane associates with the TC. We can distinguish this distribution from the electron-dense region of the TC and from LR. We are not able to specify the antibody more closely because the preservation of the membranes is weak and the indirect antibody technique causes separation of the visualized gold from the antibody.

In our initial identification of the SP we used the term spanning protein to designate the unit that joins the two membranes. We consider it likely that the junctional proteins that comprise the full triadic structure include other constituents both in T-tubules and in TC. Using an SP affinity column is one way to explore the specific association of proteins. By this means we have designated three proteins, including calsequestrin, that may constitute a part of the full junctional assembly. We are using this technique and others in an attempt to describe the junctional architecture more fully.

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References

1. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein dye binding. *Anal. Biochem.* 72:248–254.

2. Brunschwig, J.-P., N. Brandt, A. H. Caswell, and D. S. Lukeman. 1982. Ultrastructural observation of isolated intact and fragmented junctions of skeletal muscle by use of tannic acid mordanting. *J. Cell Biol.* 93:533–542.

3. Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels of unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 12:195–203.

4. Cadwell, J. J. S., and A. H. Caswell. 1982. Identification of a constituent of junctional feet linking terminal cisternae to transverse tubules in skeletal muscle. *J. Cell Biol.* 93:543–550.

5. Campbell, K. P., C. Franzini-Armstrong, and A. E. Shamoo. 1980. Further characterization of light and heavy sarcoplasmic reticulum vesicles. Identification of the "sarcoplasmic reticulum feet" associated with heavy sarcoplasmic reticulum. *Biochim. Biophys. Acta.* 602:97–116.

6. Campbell, K. P., D. H. MacLennan, and A. O. Jorgensen. 1983. Staining of the Ca^{2+}-binding proteins, calsequestrin, calmodulin, troponin C, and S-100, with the cationic carbocyanine dye "Stains-all". *J. Biol. Chem.* 258: 11267–11273.

7. Caswell, A. H., and J.-P. Brunschwig. 1984. Identification and extraction of proteins that compose the triad junction of skeletal muscle. *J. Cell Biol.* 99:929–939.

8. Caswell, A. H., Y. H. Lau, and J.-P. Brunschwig. 1976. Ouabain-binding vesicles from skeletal muscle. *Arch. Biochem. Biophys.* 176:417–430.

9. Corbett, A. M., A. H. Caswell, N. R. Brandt, and J.-P. Brunschwig. 1985. Determinants of triad junction reformation: identification and isolation of an endogenous promotor for junction reformation in skeletal muscle. *J. Membr. Biol.* 86:267–276.

10. Corbett, A. M., A. H. Caswell, R. M. Kawamoto, F. Lugo-Gutierrez, and J.-P. Brunschwig. 1985. Interaction of glyceraldehyde phosphate dehydrogenase and aldolase with microsomal subfractions of skeletal muscle. *Biophys. J.* 47:450a.

11. Eisenberg, B. R., and R. S. Eisenberg. 1982. The T-SR junction in contracting single skeletal muscle fibers. *J. Gen. Physiol.* 79:1–19.

12. Ferguson, D. G., H. W. Schwartz, and C. Franzini-Armstrong. 1984. Subunit structure of junctional feet in triads of skeletal muscle: a freeze-drying, rotary-shadowing study. *J. Cell Biol.* 99:1735–1742.

13. Franzini-Armstrong, C. 1970. Studies of the triad. I. Structure of the junction in frog twitch fibers. *J. Cell Biol.* 47:488–499.

14. Franzini-Armstrong, C. 1975. Membrane particles and transmission at the triad. *Fed. Proc.* 34:1382–1389.

15. Galfre, G., and C. Milstein. 1981. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.* 73:3–46.

16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage-T4. *Nature (Lond.).* 277:680–685.

17. McCombs, R. M., M. Benyesh-Melnick, and J.-P. Brunschwig. 1966. Biophysical studies of vesicular stomatitis virus. *J. Bacteriol.* 91:803–812.

18. Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science (Wash. DC).* 211:1437–1438.

19. Muir, A. R. 1960. The molecular structure of isolated and intracellular ferritin. *Quart. J. Exp. Physiol.* 45:192–201.

20. Saito, A., S. Seiler, A. Chu, and S. Fleischer. 1984. Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. *J. Cell Biol.* 99:875–885.

21. Somlyo, A. V. 1979. Bridging structures spanning the junctional gap at the triad of skeletal muscle. *J. Cell Biol.* 80:743–750.

22. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.