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Cytochemical, Histological, and Phylogenetic Distribution of a 38,000-Dalton Protein Associated With Transverse Tubules

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A major protein in detergent extracts of skeletal muscle appears at 38,000 daltons in electrophoretic separations. Previous investigations have provided indirect evidence that a 38-kD skeletal muscle protein is membrane associated, and this inference has served as the basis for speculations on 38-kD protein function. In the present study, affinity purified, polyclonal antisera against 38-kD protein from skeletal muscle are produced for immunolocalization and biochemical assays. Immunoblots of two-dimensional electrophoretic separations show that this protein is heterogenously charged at pI ~6.4. This 38-kD protein is not extracted from muscle in low ionic strength or high ionic strength buffers, in isotonic buffers from pH 4 to pH 8 or in buffers containing 5 mM EGTA. The 38-kD protein is extracted, however, by isotonic, pH 7.0 buffer containing 1.0% Triton-X. Light microscope observations using indirect immunofluorescence of anti-38-kD labeled tissue show the protein distributed in a reticular pattern within cross-sectioned muscle but not at the cell surface. Longitudinal sections show the protein concentrated in periodic, transverse bands. Purified fractions of muscle plasma membrane analyzed by immunoblotting contain 38-kD protein. Immunoblots using anti-38 kD show that this protein is present in all vertebrate skeletal muscle examined, however, the protein is present only in cardiac muscle that contains transverse tubules. The antibody does not recognize aldolase, another 38-kD striated muscle protein.

Key words: skeletal muscle, cardiac muscle, sarcolemma, cell membrane

In an earlier study [1], we investigated the contribution of proteins found at the surface of skeletal muscle to the mechanical energy storage capabilities of muscle cells. In that study, proteins were removed from the surface of skeletal muscle cells for comparison to normal muscle cells using collagenase and hyaluronidase digestion followed by microdissection. This treatment yielded single, contractile muscle cells that were depleted of basement membrane as evaluated by electron microscopy. One of the major proteins of muscle that was removed by this procedure was a band appearing at 38,000 daltons (D) or occasionally as a 37/39-kD doublet in one dimensional, electro-

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phoretic separations by molecular mass. Since completing these earlier studies, we have attempted to determine which depleted proteins, including the 38-kD protein, were known molecules. Although many of these proteins were found to be expected components of the basement membrane (e.g., fibronectin, laminin, collagen Type IV), the 38-kD protein has been more difficult to identify.

Several previously published studies have noted a 38-kD membrane-associated protein in skeletal muscle that could conceivably be that which was removed in our earlier analysis. One group of these previous studies [e.g., 2], reports that a 38-kD skeletal muscle protein, appearing occasionally as a 37/38-kD doublet, co-purifies with the 260-kD sodium channel at an approximate molar ratio of 1:1. These investigators have concluded that the 38-kD protein is a functional part of the sodium channel, called the β-subunit.

More recently, a second group of researchers [3] noted that a 38-kD protein from skeletal muscle displayed an affinity in vitro for a large protein (called spanning protein) found at transverse (T) tubule-sarcoplasmic reticulum junctions (triads). These investigators concluded that the 38-kD protein is located at triads and cite unpublished data that indicate the 38-kD protein is an aldolase.

Thus far, all data concerning location and function of 38-kD protein in skeletal muscle have been obtained through indirect observations either according to susceptibility to enzymatic treatment or affinity for other proteins in vitro. The goals of the present study are to obtain direct evidence for the location of 38-kD protein in vivo and to evaluate possible functions through use of immunological probes in microscopic localization and biochemical analysis.

METHODS

Electrophoresis and Immunoblotting

Skeletal and cardiac muscle from adult frogs (*Rana pipiens*; either sex), chickens (*Gallus domesticus*), mice (*Mus muscularis*; Balb C), and rabbits (New Zealand White) were removed by dissection and then separately homogenized in 10 volumes of 50 mM Tris buffer at pH 7.5 containing 150 mM sodium chloride and 0.1% sodium azide (buffer A). The samples were then boiled in sample buffer (80 mM Tris pH 6.8 containing 1.5% dithiothreitol and 2% sodium dodecyl sulfate [SDS]) and centrifuged to remove nonsolubilized tissue. The supernatant was then separated by SDS-polyacrylamide gel electrophoresis (PAGE), which contained 12% acrylamide and 0.13% bisacrylamide [4]. Molecular weight markers run in an adjacent lane were myosin (200-kD), β-galactosidase (130-kD), phosphorylase (95-kD), bovine serum albumin (BSA; 68-kD), and ovalbumin (42-kD). Some gels were stained with Coomassie blue and other, identically loaded gels were electrophoretically transferred to nitrocellulose sheets [5]. Transfers were made at 0.8 amps for 2 hr at ~4°C. Following transfer, the nitrocellulose was washed in buffer A containing 0.2% gelatin, 3% BSA, and 0.05% Tween-20. The nitrocellulose sheets were incubated with affinity purified, anti-38-kD diluted 1:10 in buffer A containing 0.25% inactivated horse serum, 0.2% gelatin, and 0.05% Tween-20. The nitrocellulose sheets were incubated with affinity purified, anti-38-kD diluted 1:10 in buffer A containing 0.25% inactivated horse serum, 0.2% gelatin, and 0.05% Tween-20. The nitrocellulose sheets were then extensively washed in buffer A to which 0.2% gelatin and 0.05% Tween-20 were added and then incubated in ^{125}I-goat, anti-rabbit, IgG for 1 hr at room temperature. This radiolabeled antibody was prepared by iodination using the

chloramine T method [6] and had an activity of 2×10^6 cpm/ml. The second antibody solution contained 2% hemoglobin, 0.2% gelatin, and 0.05% Tween-20 in buffer A. The nitrocellulose sheets were finally washed, air dried, and exposed for autoradiography.

Two-dimensional (2-D) electrophoretic separations of samples were conducted according to O'Farrell [7]. Samples were prepared for 2-D separations by homogenizing 200 mg of frozen hind limb muscle from frog in 300 μl of sample buffer. The sample was then frozen-thawed several times in liquid nitrogen before applying to the first dimension for separation by charge.

Protein Separation

Unstained gels of frog semitendinosus muscle fractions were immersed in 10 volumes of sodium acetate solution to visualize protein bands [8]. A band at 38-kD was cut out with a razor blade, briefly rinsed in deionized water, and then homogenized in 20 volumes of deionized water containing 100 μM phenylmethylsulfonyl fluoride. The homogenate was stirred at 7°C for ~17 hr and then centrifuged to remove gel fragments. The supernatant was dialyzed against 500 volumes of deionized water for 6 hr at 7°C and then lyophilized. Purity of the sample was checked by SDS-PAGE.

Antibody Production

The lyophilized, frog skeletal muscle 38-kD protein (~20 μg) was emulsified in Freund's complete adjuvant and used to inoculate a rabbit both intramuscularly and subcutaneously. Samples of pre-immune serum were drawn from the rabbit before inoculation with the antigen. One month following the first inoculation, the animal was given a booster injection of ~20 μg of 38-kD protein in Freund's adjuvant. One week later, anti-serum was drawn from the rabbit. Specificity and reactivity of the antiserum to the 38-kD protein were assayed by applying a series of dilutions of the antiserum in buffer A containing 0.2% gelatin, 0.05% Tween-20, and 5% vol/vol inactivated horse serum to nitrocellulose immunoblots of muscle extracts. Another set of blots were similarly treated with an identical dilution series of the pre-immune serum. Blots were treated with sera for 90 min at 20°C and then washed several times in buffer A containing 0.2% gelatin and 0.05% Tween-20 followed by an overnight wash at 4°C. The blots were then treated with ^{125}I-affinity purified, goat anti-rabbit IgG for 1 hr at room temperature. After being washed extensively, the blots were air dried and autoradiographed.

Antibody Purification

Our technique for purification of antisera is based upon the procedure described by Cox et al. [9]. A preparatory SDS-acrylamide gel loaded with ~1 mg of frog muscle homogenate was used for one-dimensional electrophoresis. Following protein separation, a single lane was sliced out of the gel and placed in Coomassie blue stain for 10 min. The remainder of the gel was placed in buffer A at 4°C. The stained lane was then destained and placed in water until it swelled to the same length as the unstained gel. Using the stained gel as a guide, a strip was cut out of the unstained gel at 37 to 38 kD. Protein in the 37–38-kD strip was then electrophoretically transferred to a nitrocellulose sheet at 0.8 amps for 2 hr. The remainder of the gel was stained with Coomassie blue to confirm that the gel slice contained only a 37-38-kD strip.

The nitrocellulose was then washed in buffer A containing 0.2% gelatin, 0.05% Tween-20, and 3% BSA for 2 hr at 4°C on a tissue rotator. The nitrocellulose was cut into 4-mm^2 pieces and incubated overnight in anti-38-kD serum diluted 1:5 in buffer A

containing 0.2% gelatin, 0.05% Tween-20, 2.5% BSA, and 0.25% heat-inactivated horse serum at 4°C on a tissue rotator. The nitrocellulose pieces were then rinsed five times for 10 min in buffer A containing 0.2% gelatin and 0.05% Tween-20. The pieces were placed in a syringe and eluted with 4 ml of 0.2 M glycine-HCl at pH 2.8 for 2 min with agitation. The glycine-HCl was then emptied into a beaker and quickly neutralized with 50 mM Tris-HCl pH 9.0. Sodium azide was added to 0.02 M and BSA to 0.5%.

Immunohistochemistry

Frog semitendinosus muscles were exposed by dissection and fixed at physiological length with 3.8% formalin in 15 mM sodium phosphate buffer at pH 7.0 containing 150 mM NaCl. The muscles were then cut longitudinally into strips and dehydrated in a graded series of ethanols, cleared in xylene and embedded in paraffin. Muscles were sectioned at ~10 μm, and the sections deparaffinized and hydrated. Sections were rinsed in buffer A containing 0.05% Tween-20, 0.2% gelatin, and 0.3% BSA for 20 min and then treated for 20 min at room temperature with either (a) anti-38-kD (1:200); (b) preimmune serum; or (c) buffer A. The sections were washed in buffer A for 15 min and then in buffer A containing 0.05% Tween-20, 0.2% gelatin, and 0.3% BSA for 1 hr. The sections were then incubated in fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG diluted 1:200 in buffer A for 30 min at room temperature. The sections were washed in several changes of buffer A, in water, and then mounted for observation by phase and epifluorescence optics with a Leitz Ortholux microscope. Other sections were prepared identically except Tween-20 was eliminated from buffers.

Alternatively, frog skeletal muscle was frozen on a cryotome and 16-μm-thick sections were cut, mounted on glass slides, and then immunolabeled by the procedure described above.

Plasma Membrane Fractionation

Plasma membrane, including both T-tubule and surface membrane, was purified using a modification of the procedure of Seiler and Fleischer [10]. Approximately 60 gm of skeletal muscle was dissected from adult chickens and cleaned of obvious connective tissue, fat, and blood vessels. The muscle was cut into 1- to 2-cm^3 pieces with a razor blade and then blended in an Omnimixer (Sorvall) at setting 5 for 10 sec in 300 ml of 5 mM Tris-HCl pH 7.2 containing 0.75 M KCl (buffer B). The homogenate was centrifuged for 20 min at 15,000g and the supernatant discarded. The pellet was resuspended in 300 ml of buffer B and again centrifuged. The supernatant was again discarded and the pellet resuspended in 300 ml 5 mM Tris pH 7.2 containing 0.3 M sucrose. Centrifugation at 15,000g was repeated, supernatant discarded, and the pellet homogenized in 300 ml of 5 mM Tris pH 7.2 with 0.3 M sucrose. The homogenate was again centrifuged at 15,000g, the pellet was discarded and supernatant filtered through five layers of gauze. The filtrate was then centrifuged for 2 hr at 104,000g, the supernatant discarded, and the pellet was resuspended in 15 ml of buffer B using a Dounce homogenizer. The solution was maintained on ice for 1 hr. The supernatant was then divided into 5-ml aliquots and each aliquot placed on a discontinuous gradient of 5 ml 17% (wt/wt) sucrose in 5 mM Tris pH 7.2, 5 ml 23% sucrose in 5 mM Tris pH 7.2, and 5 ml 50% sucrose in 5 mM Tris pH 7.2. Gradients were centrifuged in swinging bucket rotors for 14 hr at 75,000g. The 17/23% interface was collected, diluted with 5 mM Tris pH 7.2, and centrifuged at 160,000g for 1 hr. Samples of the pellet were then

either fixed in 1.4% glutaraldehyde in 0.2 M cacodylate buffer followed by 1% osmium tetroxide, dehydration, and embedding for electron microscopy or they were solubilized in sample buffer and prepared for electrophoresis and immunoblotting.

Samples of transverse tubules from rabbit skeletal muscle were obtained as a gift from Dr. Philip Palade (University of Texas, Galveston, TX). The transverse tubule samples were prepared according to Lazdunski's modifications [11] of the purification technique of Rosenblatt et al. [12]. This sample was also prepared for electrophoresis and immunoblotting.

Skeletal Muscle Extractions

Frog semitendinosus muscle was removed by dissection and placed in 75 mM potassium acetate, 15 mM sodium phosphate, 5 mM K_2 ethylene-glycol-bis (β-amino-ethylether)-N,N′-tetraacidic acid, 5 mM magnesium chloride containing 2% povidone (M.W. = 40,000) at pH 7.0 [13]. Several bundles of ~10–20 cells were dissected from the muscle leaving the attachment of the cells to tendon collagen fibers at the end of the cells intact. Cell bundles were then placed in one of the following extraction solutions and washed with agitation for 30 min at 7°C: (a) 15 mM sodium phosphate containing 150 mM sodium chloride (PBS) at pH 4.0, 5.0, 6.0, 7.0, or 8.0; (b) PBS pH 7.0 containing 50 mM NaCl or 200 mM NaCl instead of 150 mM NaCl; or (c) PBS pH 7.0 containing 1.0% Triton-X. Following extraction, cells were washed three times for 5 min in PBS pH 7.0 and then prepared for electrophoresis and immunoblotting.

Aldolase Assay

A degradation product of aldolase identified in SDS-PAGE appears at ~38,000 D. To determine whether the affinity purified anti-38-kD we have produced recognizes aldolase, we first assayed to see if aldolase was present in our muscle samples at ~38-kD in SDS-PAGE separations and then tested whether the affinity purified anti-38-kD we have manufactured recognizes purified aldolase.

The assay for aldolase was based upon the technique of Susor et al. [14] except the assay was performed directly on acrylamide gels rather than on agar overlays. Samples of frog and chick skeletal muscle and purified, rabbit skeletal muscle aldolase (Sigma Chemical Co., St. Louis, MO) were separated by SDS-PAGE. Gels were washed twice for 15 min in 60 ml of 50 mM Tris-HCl at pH 8 containing 25% isopropanol and then three times for 20 min in 50 mM Tris-HCl pH 8. Gels were then incubated in the dark in 80 ml of 50 mM Tris HCl pH 7.8 containing 4.8 mM fructose 1,6 diphosphate, 6.0 mM sodium arsenate, 0.37 mM nitroblue tetrazolium, 1.3% mM β-nicotinamide adenine dinucleotide (NAD), 0.06 mM phenazine methosulfate, and 400 units of glyceraldehyde-3-phosphate dehydrogenase for 70 min and then photographed.

Other gels were run using extracts of frog, chick, and rabbit skeletal muscle as well as purified, rabbit skeletal muscle aldolase. Proteins from these gels were electrophoretically transferred to nitrocellulose and treated with affinity purified anti-38 kD and then ^{125}I-goat, anti-rabbit IgG before autoradiography.

RESULTS

Adult frog skeletal muscle boiled in reducing, SDS-sample buffer and separated electrophoretically by molecular mass contains a major protein band at 38 kD (Fig. 1). Separation of 38-kD protein from other muscle proteins by cutting the band out of the

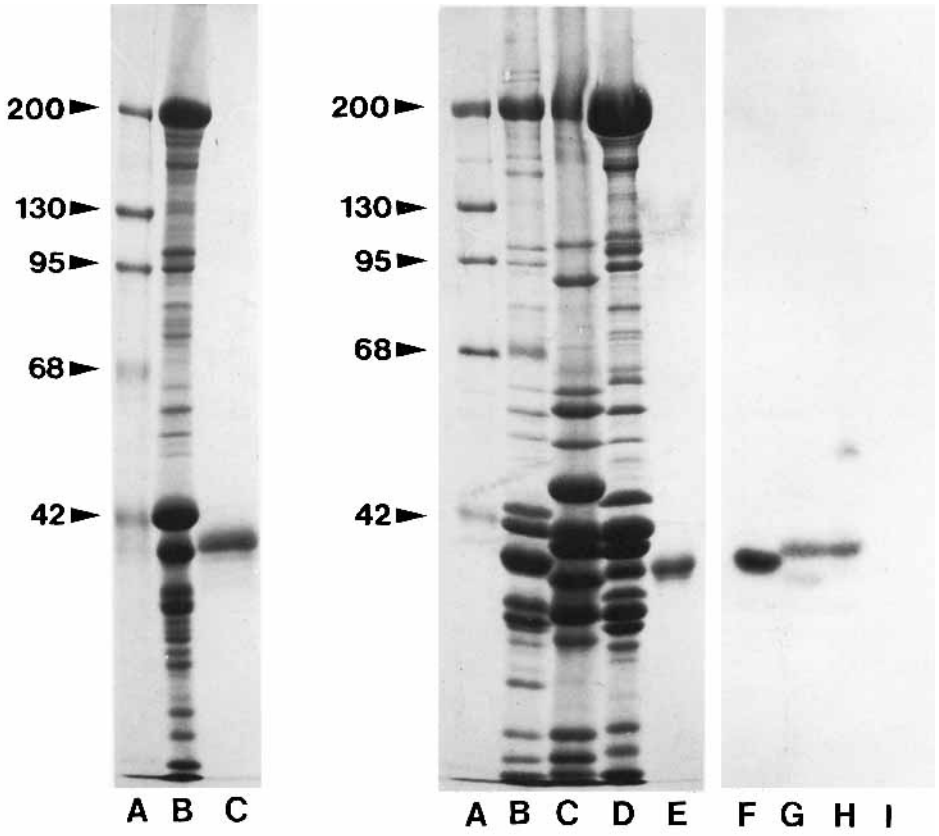

Fig. 1. SDS-PAGE separations. **A:** Molecular weight standards (myosin = 200 kD; β-galactosidase = 130-kD; phosphorylase = 95-kD; bovine serum albumin = 68-kD; ovalbumin = 42-kD). **B:** Sample buffer extract of frog skeletal muscle. **C:** Purified sample of 38-kD skeletal muscle protein.

Fig. 2. SDS-PAGE separation of molecular weight standards and muscle samples. **A:** Molecular weight standards. **B:** Frog skeletal muscle. **C:** Chicken skeletal muscle. **D:** Rabbit skeletal muscle. **E:** Purified rabbit skeletal muscle aldolase (Sigma Chemical Co., St. Louis, MO). **Lanes F–I** are immunoblots loaded identically to **lanes B–E** and incubated with affinity purified anti-38-kD. **F:** Frog skeletal muscle. **G:** Chicken skeletal muscle. **H:** Rabbit skeletal muscle. **I:** Rabbit skeletal muscle aldolase.

gel and extracting proteins in the band with water show a sample greatly enriched in 38-kD protein (Fig. 1).

Affinity purified, polyclonal antisera to 38-kD protein binds in immunoblots to 38-kD protein in frog skeletal muscle and 40-kD protein in chicken, mouse, and rabbit skeletal muscle (Fig. 2). This antiserum does not bind any protein in immunoblots of frog or chicken cardiac muscle (Fig. 3) but does bind to a 40-kD protein in immunoblots of rabbit cardiac muscle (Fig. 4). The antisera does not bind to purified rabbit skeletal muscle aldolase (Fig. 2) nor do proteins recognized by the antibody display aldolase activity (data not shown).

Two-dimensional electrophoretic separations of frog skeletal muscle proteins stained with Coomassie blue show that nearly all 38-kD protein appears at an elliptical spot at

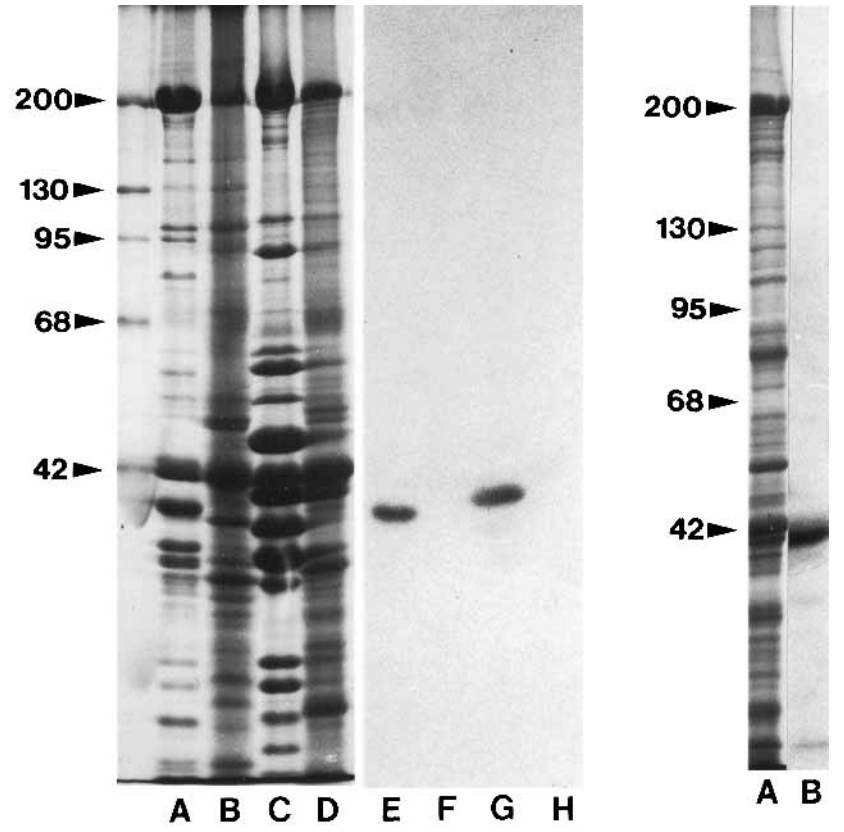

Fig. 3. SDS-PAGE separations and immunoblots of striated muscle. **A:** Frog skeletal muscle. **B:** Frog cardiac muscle. **C:** Chicken skeletal muscle. **D:** Chicken cardiac muscle. **Lanes E–H** are immunoblots loaded identically to **lanes A–D** and incubated with affinity purified anti-38-kD. **E:** Frog skeletal muscle. **F:** Frog cardiac muscle. **G:** Chicken skeletal muscle. **H:** Chicken cardiac muscle.

Fig. 4. **A:** SDS-PAGE separation of rabbit cardiac muscle. **B:** Immunoblot of rabbit cardiac muscle with anti-38-kD.

pI ~6.4 (Fig. 5). Immunoblots of 2-D gels of frog skeletal muscle using anti-38 kD show the antibody recognizes only 38-kD protein at pI ~6.4 (Fig. 6).

Fractionation of chicken skeletal muscle by modification of a previously described procedure [10] for purifying muscle plasma membrane yields a pellet containing vesiculated membrane (Fig. 7). Immunoblotting with affinity purified anti-38-kD shows these membrane pellets contain 38-kD protein (Fig. 8). Purified T-tubule membrane from rabbit skeletal muscle also contains a ~38-kD protein demonstrated in immunoblots using affinity purified, anti-38-kD (Fig. 8). The difference in apparent molecular weight of the antigen in whole rabbit skeletal muscle extracted in SDS-containing, reducing buffer (40-kD) and in purified T-tubule samples (38-kD) may result from modification of the protein by proteolysis or deglycosylation during T-tubule purification.

Extraction of frog skeletal muscle cells with low ionic strength, high ionic strength, acidic, and basic buffers shows no depletion in 38-kD protein relative to other muscle

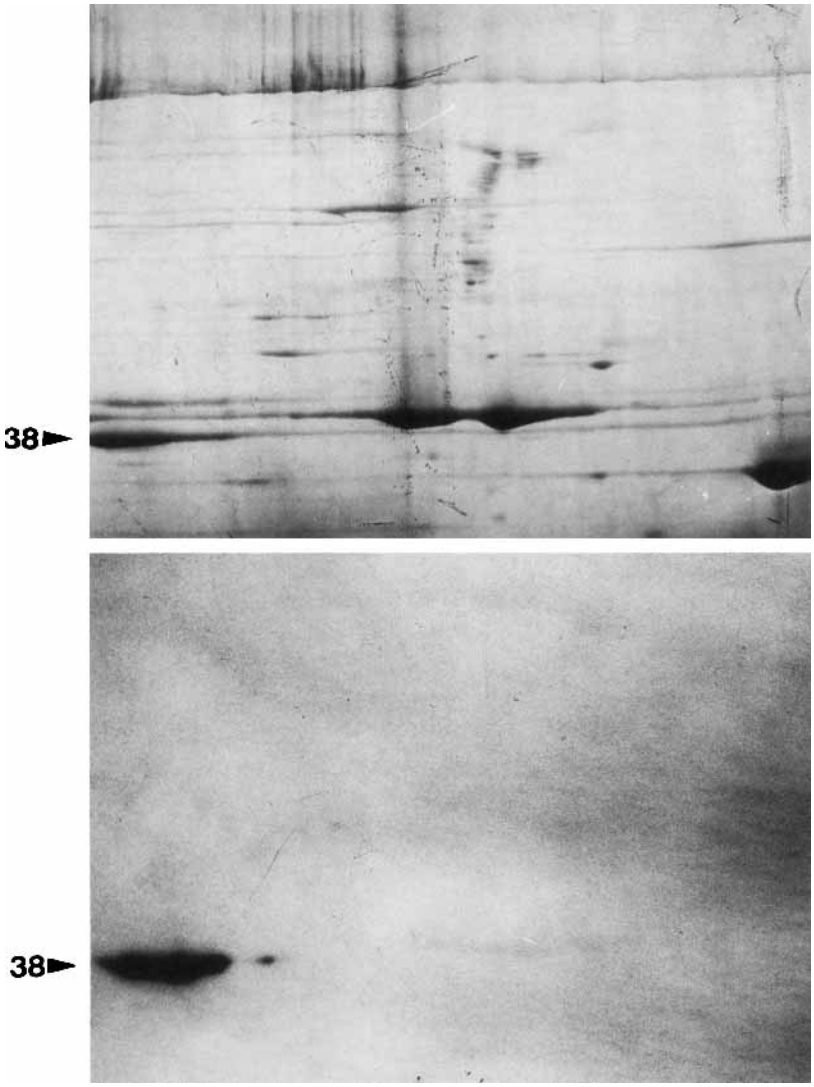

Fig. 5. Two-dimensional electrophoretic separation of proteins extracted from frog skeletal muscle. Note that nearly all protein at 38-kD is near the basic end (left side) of the gel, focusing at an elliptical spot at pI ~6.4.

Fig. 6. Immunoblot using anti-38-kD against 2-D gel of proteins extracted from frog skeletal muscle.

proteins evaluated by electrophoresis and immunoblotting (Fig. 9). Extraction procedures using detergent, however, reduce the concentration of 38-kD protein in muscle (Fig. 9). Previous investigations have shown depletion of 38-kD protein by hyaluronidase/collagenase digestion [1].

Indirect immunofluorescence of cross-sections of single skeletal muscle fibers from frog semitendinosus labeled with affinity purifed anti-38-kD and fluorescein-conjugated goat anti-rabbit IgG show fluorescence in a reticular pattern within the labeled cells (Fig. 10). Closer examination shows that there is no fluorescence at the cell surface and that

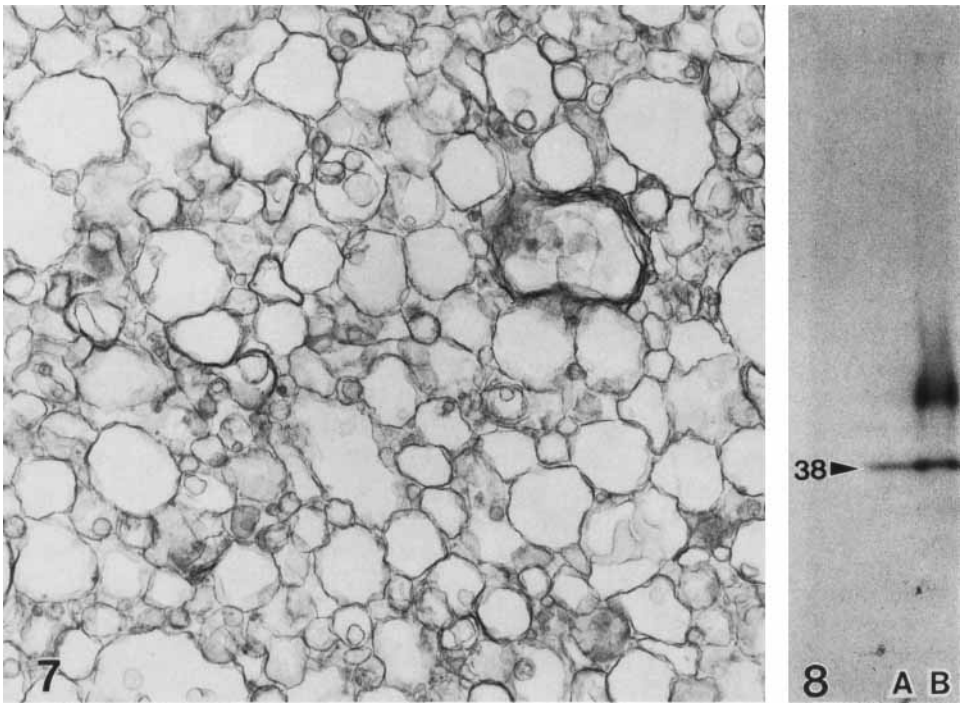

Fig. 7. Transmission electron micrograph of plasma membrane pellet of chicken skeletal muscle. Bar = 0.5 μm.

Fig. 8. Immunoblot using affinity purified anti-38-kD applied to sample containing: **A,** chicken skeletal muscle plasma membrane pellet, and **B,** rabbit skeletal muscle transverse tubules. Higher molecular weight band in lane B is protein recognized by iodinated, goat anti-rabbit IgG second antibody.

bands of fluorescently labeled material extend from the cell surface into the cell (Fig. 11). No fluorescence was seen in sections treated similarly with preimmune serum or buffer only in place of anti-38-kD (data not shown).

Longitudinal, frozen sections labeled with anti-38-kD antibody for indirect immunofluorescence show the antigen concentrated in transverse bands within the cells that coincide with Z-disc periodicity (Fig. 12). This labeling does not occur if sections are rinsed in Tween-20 containing buffer before immunolabeling.

DISCUSSION

In several previous studies, investigators have inferred from indirect observations that a 38-kD protein is associated with skeletal muscle membranes. For example, it was noted in one study that 38-kD protein has an in vitro binding affinity of 1:1 stoichiometry with a known membrane protein, the sodium channel [2]; another reported that 38-kD protein has an in vitro binding affinity for a triad protein [3]; a final study reported that 38-kD protein is susceptible to collagenase/hyaluronidase degradation on the surface of intact skeletal muscle cells and may be one of a group of proteins contributing to energy dissipation in muscle [1]. Although no direct data concerning distribution of 38-kD proteins were available in any of these studies, it is implied that 38-kD protein is

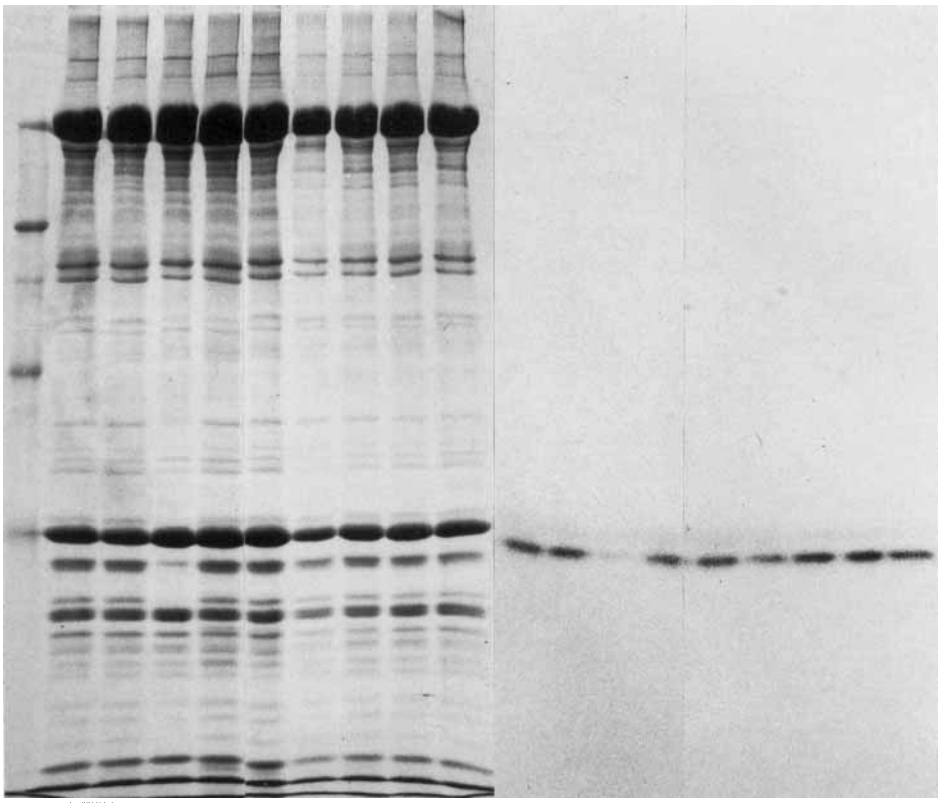

Fig. 9. SDS-PAGE separation of molecular weight standards **(A)** and frog skeletal muscle extracted with the following: **B,** PBS pH 7.0 with 50 mM NaCl; **C,** PBS with 200 mM NaCl; **D,** PBS with 1% Triton-X; **E,** PBS with 0.5 mM EDTA; and **F–J,** PBS with 150 mM NaCl at pH 4.0, pH 5.0, pH 6.0, pH 7.0, and pH 8.0. **Lanes B′ to J′** are autoradiographs of immunoblots of identical samples as **lanes B to J** incubated with anti-38-kD.

distributed along muscle cell surface membrane and T-tubules [2], at triads [3], or at surface membrane only [1]. Possible interpretations for these diverse data include: (1) there is more than one 38-kD membrane-associated protein in muscle or (2) there is one major 38-kD protein, and some inferences regarding its distribution, and possibly its function, are incorrect.

An earlier study has shown that extracts of skeletal muscle in SDS reducing buffer separated according to molecular mass by SDS-PAGE contains a prominent band at 38-kD, which appears as a 37/39-kD doublet in some gels [1]. This 38-kD protein binds wheat germ agglutinin (WGA), gives a positive reaction with periodic acid-Schiff reagent for vicinyl diglycols, and is removed by incubation of intact cells with collagenase/hyaluronidase solution [1]. These data all indicate that 38-kD protein is a cell surface glycoprotein. The findings reported in the present study that 38-kD protein is not extracted from muscle cells by high ionic strength, low ionic strength, basic, or acidic buffers but is extracted by detergent indicate that the protein is an integral membrane protein or linked to an integral membrane protein.

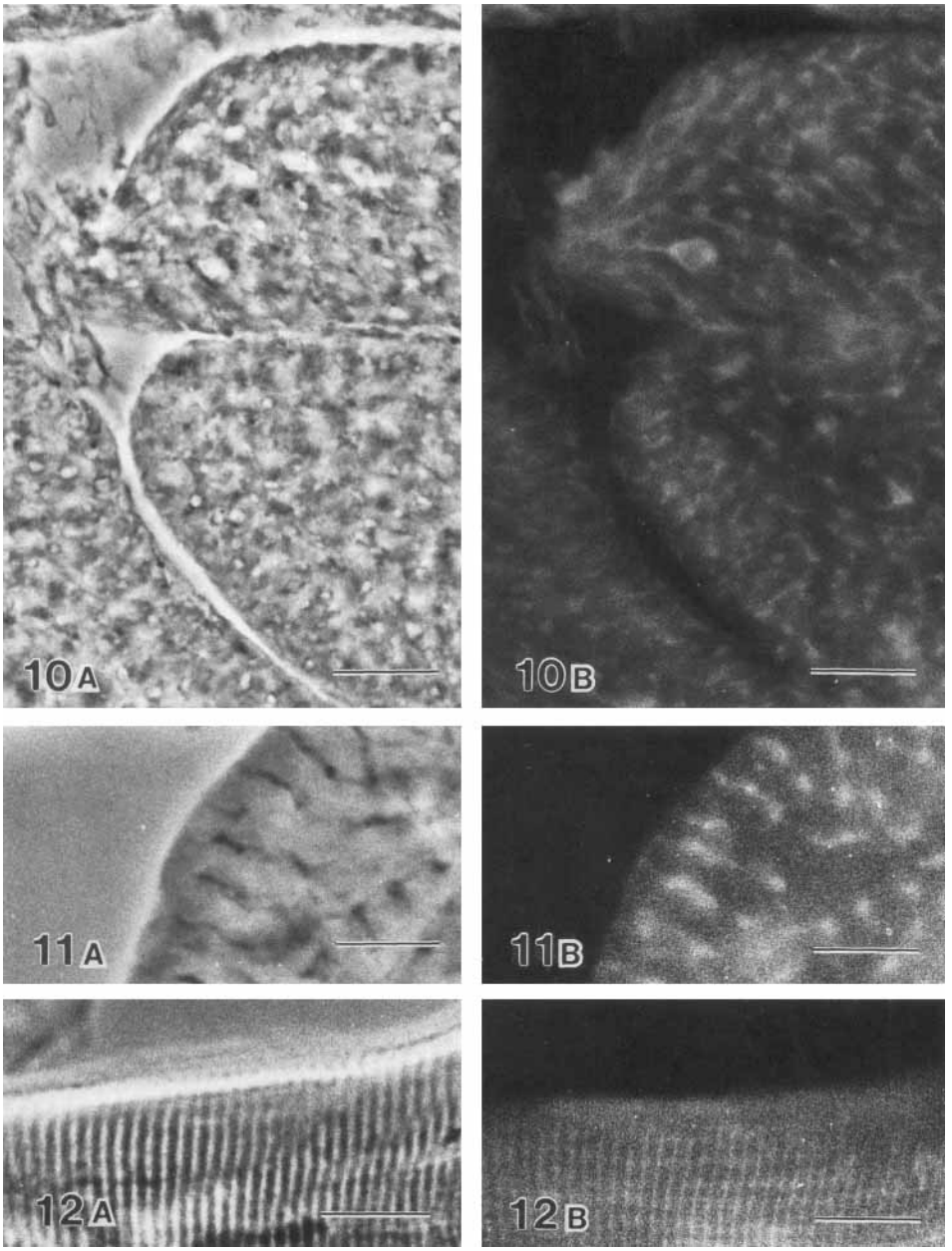

Fig. 10. Cross-section of paraffin-embedded frog skeletal muscle cells. **A:** Phase micrograph. **B:** Same section as 10A labeled with affinity purified anti-38-kD and FITC-conjugated, goat anti-rabbit IgG. Note reticular pattern of fluorescence within the cells. Bar = 8 μm.

Fig. 11. Cross-section of surface of one paraffin-embedded frog skeletal muscle cell. **A:** Phase micrograph. **B:** Indirect immunofluorescence of same field as 11A labeled with anti-38-kD. Note that bands of fluorescence extend from cell surface into the cell yet no fluorescence is apparent at the surface of the cell. Bar = 2.5 μm.

Fig. 12. Longitudinal section of frozen section of frog skeletal muscle incubated with affinity purified anti-38-kD and FITC-second antibody and viewed by: **A,** phase microscopy, and **B,** epifluorescence. Bar = 20 μm.

Two observations reported here further indicate that the 38-kD glycoprotein observed in SDS-PAGE separations of frog skeletal muscle is a single glycoprotein. First, isoelectric focusing shows that the protein recognized by anti-38 kD appears as a single elliptical spot at pI ~6.4. The elliptical shape of the spot indicates some charge heterogeneity, which is expected of glycoproteins. Thus, the appearance of all observable 38-kD protein over a narrow range of isoelectric points is consistent with the existence of one major 38-kD glycoprotein. Second, immunohistochemical and biochemical data show that antisera to 38-kD protein recognize an antigen at T-tubules, yet not at the surface membrane or elsewhere in the muscle or surrounding connective tissue. This specific localization is also consistent with the existence of a single major 38-kD glycoprotein in skeletal muscle.

The appearance of 38-kD protein in one-dimensional, SDS-PAGE separations of whole muscle extracts indicates that 38-kD protein is a major component of skeletal muscle. However, it is noteworthy that not all proteins are comparably soluble in the muscle extraction procedure we use in which tissue is briefly boiled in a reducing, detergent-containing buffer near neutral pH. Some material remains insoluble in this procedure. However, this procedure is thought to be very effective at extracting membrane proteins. We expect, therefore, that membrane proteins are greatly enriched in this sample.

The conclusion that the major 38-kD protein in frog skeletal muscle is a T-tubule-associated protein gains further support through data presented here concerning the histological and phylogenetic distribution of 38-kD protein. Skeletal muscle from all vertebrates examined (frog, chicken, mouse, and rabbit) contain ~38-kD protein that is recognized by anti-frog skeletal muscle 38-kD. This indicates that this protein is well-conserved in vertebrate muscle. Cardiac muscle from frog and chicken do not contain this protein, although cardiac muscle from rabbit does contain ~38-kD protein recognized by this antibody. At first view, it seems anomalous that anti-frog skeletal muscle 38-kD recognizes a ~38-kD protein in the heart of one phylogenetically remote vertebrate (rabbit) but not in that of a similarly remote (chicken) or identical (frog) vertebrate. This, however, is consistent with the protein localization reported in this study. Mammalian cardiac tissue contains T-tubules while avian and amphibian hearts do not [15]. Thus, there is a 1:1 correlation between the occurrence of T-tubules and the presence of 38-kD protein in all vertebrate tissues analyzed in immunoblots.

These observations are adequate to justify rejecting the hypothesis implied in an earlier study [1] that 38-kD protein was one of several proteins located at the muscle cell surface that contribute to energy dissipation in skeletal muscle. Two further observations also indicate that the 38-kD T-tubule-associated protein studied here is distinct from the 38-kD T-tubule-associated aldolase reported by Kawamoto et al. [3]. First, the rabbit skeletal muscle protein that is antigenically similar to the 38-kD frog skeletal muscle protein occurs at ~40 kD in reducing gels. Thus, in rabbit tissue, the protein studied in this investigation and that noted by Kawamoto et al. [3] have different molecular mass in SDS-PAGE. Second, the rabbit skeletal muscle protein recognized by anti-frog skeletal muscle 38-kD does not display aldolase activity, whereas Kawamoto et al. [3] cite unpublished data to indicate that the rabbit 38-kD protein with binding affinity for a triad protein is aldolase.

The possibility that the 38-kD protein associated with sodium channels is the same as that localized here is intriguing. The occasional appearance of 38-kD protein studied here as a 37/39-kD doublet is a characteristic shared with the 38-kD skeletal muscle

protein that some investigators consider a subunit of the sodium channel. The presence of these subunits has been considered controversial in some tissues [2], although current evidence suggests a functional association between the 260-kD sodium channel and 38-kD protein. This evidence includes the following. First, WGA affinity chromatography yields a 38-kD protein in addition to the 260-kD sodium channel from rat brain with a stoichiometry of 1:2 for 260-kD:38-kD following affinity purification [16]. This suggests a relationship between 38-kD protein and the 260-kD sodium channel. Second, the stochiometry of 260-kD:38-kD protein from rabbit T-tubules is 1:1, which suggests functional association between these proteins, at least during chromatographic procedures [2]. Third, affinity purification of proteins from rat skeletal muscle using either monoclonals or polyclonal antibodies to the 260-kD sodium channel protein also yield a 38-kD protein or 37/39-kD doublet [2]. Finally, affinity purification of muscle or brain proteins using tetrodotoxin (TTX) or saxitoxin (SX) binding as part of the purification procedure also yields 37/39-kD or 38-kD protein [16,17], although evidence indicates that TTX and SX bind only to the 260-kD sodium channel protein [18–20]. These studies have led several groups of investigators to conclude that the 38-kD protein is a subunit of the sodium channel, called the β-subunit [e.g., 2,16,21,22].

Although the 38-kD protein may be a subunit of the sodium channel, its presence is not necessary for channel function [2]. For example, channels purified from eel demonstrate normal, voltage-dependent action even though no β-subunits are present [23]. This has led several investigators to conclude that the presence of 38-kD protein modifies channel function [2,16], which would provide an explanation why the 38-kD protein cannot be demonstrated in all excitable membranes containing Na channels.

The present study shows that the distribution of 38-kD protein is restricted to T-tubules in skeletal muscle and cardiac muscle. In animals whose hearts do not contain T-tubules, the protein is either absent or present at very low concentrations that cannot be discerned in our immunoblotting procedures. If the 38-kD protein studied here is indeed the 38-kD β-subunit, then the localization of 38-kD protein reported here is consistent with the proposal that the β-subunits modify sodium channel function because the behavior and drug-binding properties of T-tubule sodium channels differ from those of surface membrane sodium channels. Several examples of this follow.

First, frog skeletal muscle cells show two phases of early ion fluxes across the membrane that are attributable to sodium channels [24]. Physiological evidence indicates that a rapid, early inward current is attributable to surface membrane sodium channels while a slower, late inward current is atributable to T-tubule sodium channels [24]. This suggests unexplained differences in T-tubule channel molecular structure.

Second, T-tubule and surface membrane sodium channels display different drug-binding behavior. A derivative of TTX applied to frog skeletal muscle fibers binds with much higher affinity to T-tubules than to surface membrane [25], whereas the scorpion toxin Css_{II} [26] produced a partial block of surface membrane channels but had little effect on T-tubule channels.

The localization of 38-kD protein to T-tubules in the present study provides morphological evidence consistent with the structural differences between surface and T-tubule membranes inferred from physiological, biochemical, and pharmacological studies. The present findings are not sufficient to conclude that the 38-kD protein localized here and the 38-kD putative subunit of Na channels are the same protein, nor can data presented here prove that the physiological and pharmacological differences between T-tubule and surface membrane sodium channels are attributable to the presence or absence

of the 38-kD protein. They do suggest, however, that these are working hypotheses and indicate that anti-38-kD may provide a probe for these further studies.

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