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A Basement Membrane-Associated Glycoprotein From Skeletal Muscle

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We have isolated a major glycoprotein that appears to be associated with rat skeletal muscle basement membrane. We determined that the glycoprotein was part of the muscle cell surface complex when we found it to be enriched in preparations of muscle ghosts. We isolate the glycoprotein from homogenized muscle preextracted with 4 M and 8 M urea. It elutes as a major component in the presence of 8 M urea/50 mM 2-mercaptoethanol. Its apparent molecular weight on sodium dodecyl sulfate gels is 130,000. Amino acid analysis indicates that it is not a collagen but that it does contain small amounts of hydroxyproline and hydroxylysine. There may be collagenous domains in the glycoprotein molecule, for it is cleaved into three fragments by purified bacterial collagenase. Immunoperoxidase staining confirms that the 130,000-dalton protein is localized at the surface of adult skeletal muscle cells. It is probably a general basement membrane-associated glycoprotein because we found material immunologically cross-reactive with the muscle glycoprotein in basement membrane regions of kidney, liver, brain, and small intestine. We have shown the glycoprotein to be distinct from fibronectin, laminin, and types I, III, IV, and V collagens in enzyme-linked immunosorbent assays.

Key words: basement membrane, extracellular matrix, muscle, structural glycoprotein

The surface components of adult skeletal muscle cells in vivo are arranged in a series of structures best discerned by electron microscopy. The surface complex consists of a plasma membrane with a carbohydrate-rich coat, a felt-like electron dense basal lamina, and an overlying reticular lamina containing collagen and reticular fibrils embedded in an amorphous matrix [1]. Together the basal and reticular laminae make up the basememt membrane. The outer limit of muscle basement membrane is poorly defined as the reticular lamina merges into the supporting endomysial connective tissue network. Scanning electron microscopy of the muscle surface shows a layer of fibrils surrounding each myotube, and, perpendicular to this layer, collagen fibrils anchor capillaries to the myotubes and myotubes to each other [2].

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N′-tetraacetic acid; Tris, Tris (hydroxymethyl) aminomethane; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Con A, concanavalin A; PAS, periodic acid–Schiff reagent; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PAP, peroxidase antiperoxidase.

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Attempts to isolate or characterize muscle components situated external to the plasma membrane have met with only limited success. Fibronectin, laminin, and collagens have been isolated from other tissues, and antibodies raised against these proteins have been used to detect them in muscle basement membrane [3–5]. Additional muscle surface antigens have been recognized, but none has been characterized [6, 7]. We have set out to isolate components associated with adult skeletal muscle cell surfaces. We have found a major extracellular glycoprotein in skeletal muscle and have purified it [8]. The glycoprotein has an apparent molecular weight of 130,000 and is associated with the basement membrane surrounding each myotube. The amino acid composition of the glycoprotein indicates that it is not collagen per se, but it is fragmented by bacterial collagenase. We found no immunological cross-reactivity between the glycoprotein and fibronectin, laminin, or types I, III, IV, and V collagen. The glycoprotein is probably not confined to muscle since immunohistochemical techniques using antibodies raised against it reveal staining of basement membrane regions in other tissues.

MATERIALS AND METHODS

Muscle Ghosts

Muscle ghosts were prepared beginning with 15–20 gm of leg and pelvic muscle from 200- to 300-gm female Sprague-Dawley rats by a procedure modified from McCollester's original description [9, 10]. Dissected muscles were placed in a beaker of ice-cold 0.15 M NaCl. Chilled pieces were cleaned of obvious connective tissues, fat, major vessels, and nerves, and then placed in fresh ice-cold 0.15 M NaCl. Cleaned muscle pieces in suspension were minced with scissors; the mince was washed four times with cold 50 mM $CaCl_2$. The tissue was allowed to settle for 1 min after each of the first three washes and for 15 min after the last wash. The washed mince was homogenized with 60 ml of cold 50 mM $CaCl_2$ for 10 sec using a Waring blender in the cold. The slurry was filtered through a 350-μ mesh nylon screen; pieces that remained on the screen were rehomogenized in 60 ml of $CaCl_2$. This procedure was repeated four times and the screen was then washed with 30 ml of 50 mM $CaCl_2$. The homogenized muscle fragments were collected by centrifugation of the pooled filtrates at 200g for 30 sec at speed in a Dupont Sorvall HS-4 rotor. The myotube fragments were washed twice with 30 mM KCl/2.5 mM DL-histidine, pH 7.4, and then incubated at 37°C in 150 ml of the same solution for 30 min. The suspension was next cooled and centrifuged, and the muscle fragments were washed three times with fresh 30 mM KCl. To obtain ghosts, the myotube fragments were emptied by introducing 1,400 ml of cool 0.01 mM EGTA[1] (adjusted to pH 7.8-8.0 with 1 M Tris) followed by slow stirring for 1 hr at room temperature. Ghosts were collected by centrifugation at 1,200g for 4 min using the HS-4 rotor. They were then washed 2–3 times with buffered EGTA.

Glycoprotein Isolation

Starting with approximately 25 gm of rat leg muscle, the tissue was cleaned, minced, and washed as for isolation of ghosts. The muscle was homogenized once with 100 ml 50 mM $CaCl_2$ for 8 sec. The homogenate was centrifuged at 2,600g for 3 min and the pellet washed twice with buffered 30 mM KCl. The muscle pellet was incubated twice with 800 ml of 4 M urea/5 mM Tris/0.01 mM EGTA, pH 8.0, for

30 min at room temperature with gentle stirring. Following each incubation the insoluble residue was pelleted by centrifugation at 8,000 g for 4 min. The pellet was then treated with 300 ml of 8 M urea/0.5 M Tris, pH 8.5, resuspending by stirring at room temperature for 20 min. Fibrous aggregates and large muscle pieces were removed from the suspension by filtration through a 350-μ mesh nylon screen. The debris on the screen was washed with an additional 100 ml of 8 M urea/0.5 M Tris, pH 8.5, and then discarded. The filtered muscle pieces were pelleted by centrifugation at 8,000g for 4 min and extracted a second time with 200 ml of 8 M urea/0.5 M Tris, pH 8.5, for 20 min at room temperature. The pellet was extracted a final time with 75 ml of 8 M urea/0.5 M Tris/50 mM 2-mercaptoethanol, pH 8.5, for 30 min. The supernatant containing the glycoprotein was collected following centrifugation at 8,000g for 5 min; rare large suspended particles were removed by filtration through a 130-μ mesh nylon screen, and the supernatant recentrifuged at 112,000g for 90 min. The glycoprotein remains as a major component of the high-speed supernatant. After dialysis against water and lyophilization, the glycoprotein was further purified by preparative SDS PAGE (see below).

On some occasions, the urea/mercaptoethanol extract was chromatographed after the high-speed centrifugation step and prior to preparative electrophoresis. For this step, ion exchange chromatography employing DEAE-Sephadex (A-50 Pharmacia) in a batch procedure was introduced into the above protocol to isolate some glycoprotein for amino acid analysis. Both the ion exchanger and the muscle extract were preequilibrated for 20 hr at 4°C with 2 M urea/50 mM imidazole, pH 6.5, made from a deionized 8 M urea solution. One milligram of dry ion exchanger was used per milliliter of extract. Equilibrated exchanger was washed several additional times at room temperature with gentle shaking. The DEAE-Sephadex was then shaken gently with the extract for 2 hr at room temperature. Unbound material was aspirated following centrifugation and the exchanger was washed twice, 20 min each time. Bound proteins were eluted using 0.4 M NaCl in the 2 M urea buffer. The ion exchanger was shaken with this buffer twice for 45 min. Each elution was done with half the original mercaptoethanol extract volume. Pooled eluate was dialyzed against water, lyophilized, and used for preparative SDS PAGE.

Biochemical Analysis

Analytical SDS PAGE was done on 5.6% 100 × 150 × 1.5 mm slab gels with short 2.8% stacking gels. The gel system was that of Fairbanks et al, except that the final SDS concentrations were lowered to 0.2% [11]. Samples were solubilized for electrophoresis by heating at 100°C for at least 2 min, with 5.7 M urea, 1% SDS and 1% 2-mercaptoethanol. Gels were stained with Coomassie blue or periodic acid–Schiff reagent as described [11]. Glycoproteins in the gels with binding sites for Con A (Sigma) were localized following the procedure of Olden and Yamada [12]. Fixed unstained gels were incubated in the lectin solution and then horseradish peroxidase (Boehringer Mannheim). The site of the bound peroxidase, which is itself a glycoprotein, was visualized by incubating the gels with diaminobenzidine (Sigma) and hydrogen peroxide. The molecular weight of the glycoprotein was estimated on both 4% and 5.6% gels using myosin, β-galactosidase, phorphorylase B, bovine serum albumin, and ovalbumin as molecular weight standards. Preparative gel electrophoresis was done on 3.0-mm slabs of the same 5.6% gels without any stack. The glycoprotein was visualized in the unstained gels as a sharp clear zone after the gel

had been soaked in 4 M sodium acetate for 30–45 min [13]. The band was cut from the gel, rinsed briefly with water, and either frozen or used immediately. Glycoprotein was extracted from gel strips with numerous changes of 8 M urea/0.1 M Tris/50 mM 2-mercaptoethanol, pH 8.0, over several days. Amino acid analysis of the glycoprotein extracted from gel strips was done by Dr. Arthur Veis of Northwestern University. Samples were hydrolyzed in 6N HCl at 108°C for 20 hr in sealed, nitrogen-flushed tubes and analyzed on a Jeol 6AH analyzer with a single column program.

Protein was assayed according to a modification of Lowry's procedure using bovine serum albumin (BSA) as standard [14]. Samples containing mercaptoethanol were dialyzed before analysis. Separate blanks and standard curves correcting for interference by urea and Tris were used to measure protein in urea extracts.

The susceptibility of the 130,000-dalton glycoprotein to digestion by bacterial collagenase was tested using muscle urea/mercaptoethanol extracts. Centrifuged (112,000g) extract was dialyzed against 25 mM Tris/10 mM calcium acetate buffer, pH 7.4, overnight and then incubated with purified collagenase (Advance Biofactures Corp., Form III) in the presence of 9 mM N-ethyl maleimide at 37°C for periods ranging from 15 min to 2½ hr (see results). Twenty-four units of enzyme were used for 40 μg of extract protein (approximate weight ratio 1:80). Enzyme activity was blocked in controls and at the end of incubation with 20 mM EDTA. Samples were then dialyzed against water, lyophilized, and run on SDS gels.

Immunology

Two rabbits were immunized in the toe pads with 50 μg of isolated glycoprotein in 0.2 ml saline mixed with an equal volume of complete Freund's adjuvant containing 3 mg/ml myco. Tubercul. var. hominis. The rabbits were boosted IV with 150, 200, and 200 μg of glycoprotein in 1 ml of saline at intervals of 6 wk, 2 mo, and 3 mo, respectively. Blood was taken 1 wk after each boost. Rabbit antiserum against rat type III collagen was kindly provided by Dr. Zach Hall; serum against fibronectin, by Dr. Byron Anderson; and serum against laminin, by Dr. George Martin.

Rabbit sera were screened for antibodies to collagen by a passive hemagglutination assay. Types I, III, and V collagen were isolated from rat skin following pepsin (Sigma) digestion. Types I and III were precipitated from the digest with 4% NaCl. Type III was purified by precipitation four times at 1.65 M NaCl, and type I by precipitation three times at 2.7 M NaCl. Type V collagen was precipitated from the 4% NaCl supernatant by increasing the salt concentration to 7% and then reprecipitated by dialysis against water. All of the collagens were judged to be pure by SDS-PAGE. They were quantified with a Biuret protein assay standardized to bovine serum albumin. For the hemagglutination assay serial twofold dilutions of the sera were made with 1% heat-inactivated nonimmune rabbit serum in microtiter plates. Sheep red blood cells were tanned with 83.3 μg/ml tannic acid, washed, and mixed with an equal volume of glycoprotein or collagen at a concentration of 20 μg/ml. Agglutination was scored in microtiter plates after incubation of the washed, coated red blood cells at 37°C for 15 min.

A micro enzyme-linked immunosorbent assay (ELISA) procedure was used to check the relation of the 130,000-dalton muscle glycoprotein to collagens and basement membrane-associated glycoproteins. Ninety-six-well flat-bottom vinyl microtiter plates were coated by incubation overnight at 4°C with antigens at concentrations of 3 μg/ml in 20 mM sodium carbonate buffer, pH 9.6. Muscle glycoprotein was purified as described without DEAE chromatography and types I, III, and V collagen, also as described. Fibronectin, laminin, and type IV collagen were gifts from Drs.

Byron Anderson, George Martin and Arthur Veis, respectively. Excess binding sites in the wells were blocked by incubation with 0.1% BSA in phosphate-buffered saline (PBS). After washing the plates, antisera at dilutions of 10^{-3} to 10^{-5} in PBS with 0.05% Tween 20 were added. Antibody that bound to protein in the wells was detected using peroxidase-conjugated swine antirabbit immunoglobulin (Dako) diluted 1/1000. Incubations with antibody were at room temperature for 1 hr, washing at least six times after each incubation. Peroxidase activity was monitored with a substrate solution consisting of 1 mg orthophenyldiamine and 0.4 μl 30% hydrogen peroxide per ml of 0.1 M sodium citrate buffer, pH 4.5. The absorbance at 450 nm in each well was measured 30 min after addition of the substrate.

Control and collagenase-digested mercaptoethanol extracts were run on SDS gels, and protein bands recognized by immunoglobulins of our rabbit antiglycoprotein sera were detected by an immunoblotting procedure. At the conclusion of electrophoresis, gels were soaked for 20 min in 0.2 M sodium phosphate buffer, pH 7.0. Several sheets of saturated coarse filter paper, a nitrocellulose membrane (BA-85, Schleicher and Schuell), the SDS gel, and more filter paper were layered onto a slab gel dryer. Vacuum was applied (without heat), sealing the system and drawing buffer and proteins through the gel and onto the nitrocellulose sheet. The next day the gel was removed and the nitrocellulose was cut into strips for antibody staining. Excess protein binding sites on the nitrocellulose were blocked by incubation with a solution of 5% BSA in 0.9% NaCl/10 mM Tris-HCl, pH 7.4. The strips were then incubated with antimuscle glycoprotein rabbit serum or normal rabbit serum at a dilution of 1:100 followed by the same dilution of a peroxidase-conjugated goat anti-rabbit serum (Cappel Laboratories). Each serum was diluted with 1% BSA/10% normal goat serum/0.05% Nonidet P-40 in the Tris-saline buffer, and after each antibody incubation the strips were washed three times for 10 min with this medium. Bound peroxidase was visualized on the strips within seconds of putting them into a solution of 0.05% diaminobenzidine/0.01% H_2O_2 in Tris-saline buffer.

Peroxidase antiperoxidase (PAP) staining of fresh frozen tissue sections was done according to the method of Sternberger [15]. Briefly, the unfixed sections were treated with 3% hydrogen peroxide to inhibit endogenous peroxidase and then overlaid serially with 10% normal goat serum (Gibco) in Tris-buffered saline, pH 7.6, the rabbit antisera diluted 1/100 with 10% normal goat serum, goat antirabbit IgG (Cappel Laboratories) diluted 1/50 with 10% normal serum, and rabbit PAP complex (Cappel Laboratories) diluted 1/50 with 10% normal goat serum. Bound peroxidase was then visualized using diaminobenzidine.

RESULTS

Identification of the Major Glycoprotein Associated With Muscle Ghosts

Muscle ghosts were prepared by a modification of the hypotonic emptying procedure originally described by McCollester [9]. The procedure includes two important steps. The preliminary incubation at 37°C for 30 min in 30 mM KCl seems to allow endogenous enzymes to disrupt internal membranes so that when the preincubated muscle (see Fig. 1A) is mixed with a large volume of 0.01 mM EGTA, intracellular contents spill from the ends of the muscle segments, leaving "ghosts" (see Fig. 1B).

We monitored ghost preparations with Giemsa-stained slides and by SDS-PAGE. On slides the ghosts appear as empty cellophane-like cylinders often enmeshed by capillaries (see Fig. 1B). The muscle ghost preparations were invariably contami-

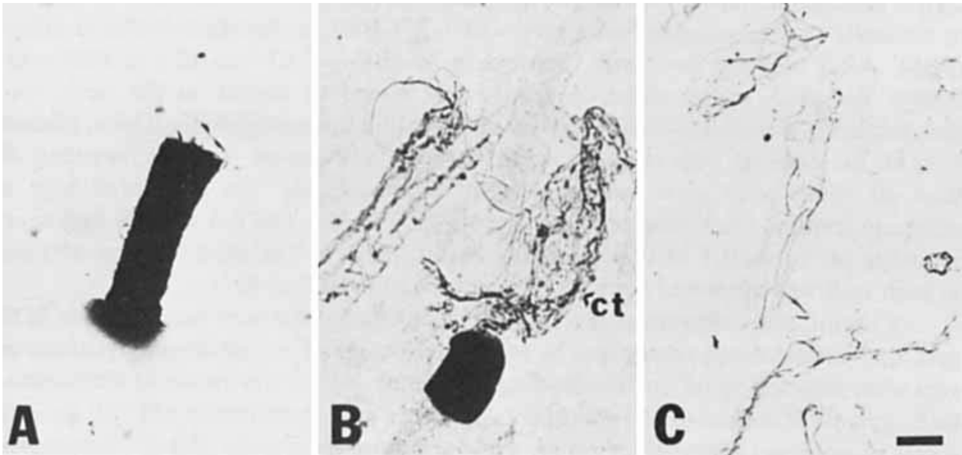

Fig. 1. Isolation and purification of muscle ghosts as seen in Giemsa-stained slides. A) The starting suspension of homogenized and filtered adult skeletal muscle consists of cylindrical myotube fragments. B) Following introduction of a large volume of 0.01 mM EGTA, the intracellular contents spill from the ends of fragments, leaving muscle ghosts—some with and some without attached capillaries. Occasional segments that have failed to empty are seen as well as rare pieces of connective tissue (ct). The stippled background staining indicates the presence of insoluble protein in ghost suspensions. C) Ghosts extracted with 4 M urea, pH 8, for 45 min show much less background protein and much less protein entrapped in ghosts. (Bar represents 100 μm.)

nated by free nuclei, nuclei within capillaries, and nuclei within the muscle ghosts themselves, as well as by rare pieces of connective tissue. Though better than 90% of the muscle fragments always spilled the bulk of their contents, a small amount of protein remained detectable within the ghosts. Most important, the patchy blue background of the Giemsa-stained slides (see Fig. 1B) indicated that insoluble proteins were pelleting together with the ghosts. Repeated washing not only failed to remove protein within or outside of the ghosts, but it also caused the muscle ghosts to stick to each other (and to the glassware), and the ghosts would no longer sediment at the speeds at which they were originally collected. The amount of the extra ghost and enclosed protein varied considerably between preparations.

When ghost preparations were solubilized with SDS and subjected to PAGE, results were consistent. Even for ghosts judged to be exceptionally clean in stained slides, the Coomassie blue protein pattern closely resembled that of the starting material, washed homogenized muscle (see Fig. 2A, B). The majority of the bands seen in ghost preparations stained with Coomassie blue were those of contractile proteins, myosin and actin comprising the major bands. Nonetheless, a significant difference between ghosts and homogenized muscle was noted when PAS or Con A staining for glycoproteins was employed. Ghost preparations were consistently enriched for glycoprotein, there being a single major glycoprotein band with an apparent molecular weight of 130,000 (see Fig. 2B). Con A staining was much more sensitive for this glycoprotein than PAS or Coomassie blue.

To confirm that the major glycoprotein was associated with muscle ghost structures and to determine where and how it was bound to the ghosts, we searched for methods to enrich for the glycoprotein and to elute it from the ghost preparations.

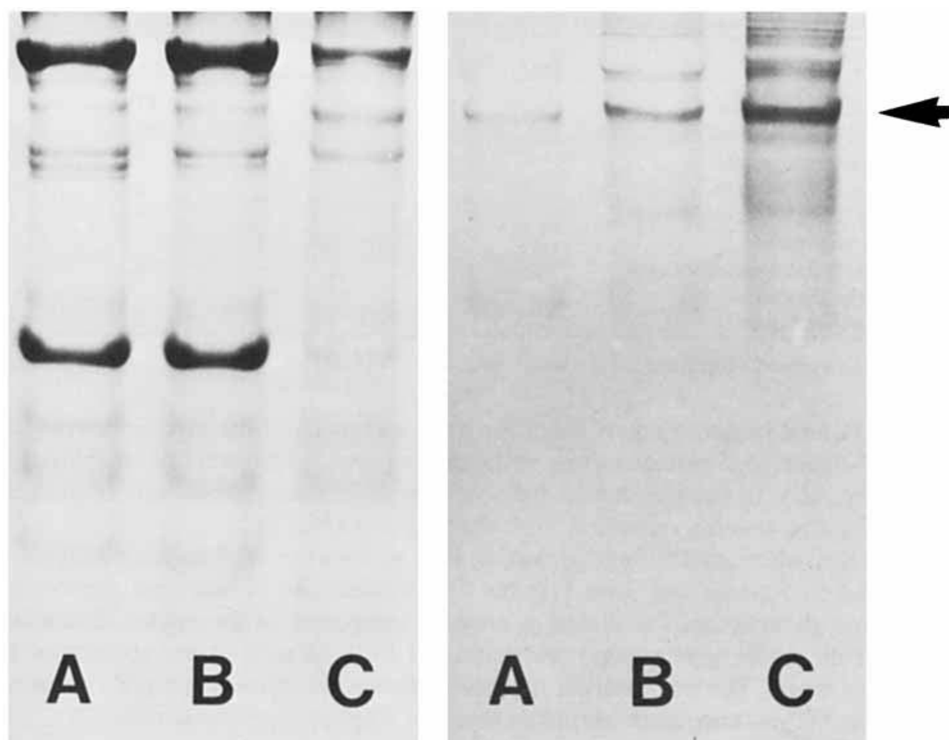

Fig. 2. SDS-PAGE of muscle ghosts. Portions of the samples shown in Figure 1 (A—washed homogenized muscle, B—muscle ghosts, C—urea-extracted ghosts) were subjected to gel electrophoresis in gels containing 5.6% polyacrylamide and 0.2% SDS and are labeled accordingly. The gels on the left were stained for protein with Coomassie blue, those on the right were "stained" for glycoprotein with Con A (see Methods). Identical amounts of the paired samples were layered on each gel, and all samples were run simultaneously. Control gels washed with α-methylmannoside after incubation with Con A did not show any staining. The two major Coomassie blue bands in the starting material correspond to myosin and actin (above and below, respectively). As the muscle ghosts were purified, these bands were depleted and there was enrichment of glycoprotein, especially the major band at 130,000 daltons (arrow). Much of the Coomassie blue stain at the major glycoprotein position in the starting material is probably not glycoprotein, because we would not expect the amount detected here with Con A to stain with Coomassie blue (see Fig. 4). In identical gels stained with periodic acid-Schiff reagent, no glycoprotein band could be seen in starting material, and only a very weak band could be seen in ghosts.

Extraction of muscle ghost preparations sequentially with the series of reagents described by Carlson et al [16] for the isolation of basement membranes from other tissues (ie, 3% Triton X-100, 50 Kunitz units/ml deoxyribonuclease in 1 M NaCl, and 4% sodium deoxycholate) was ineffective; neither the morphology nor the protein composition of ghost preparations so treated indicated an appreciable enrichment in basement membrane. Significant, also, was the fact that detergents employed for the solubilization of cell membranes did not destroy ghost structures nor did they remove the major glycoprotein from the ghosts.

Another approach toward glycoprotein purification was to find conditions for total extraction of actomyosin. Potassium chloride proved not useful because it caused aggregation of the ghosts. Urea proved more effective. Extraction of muscle ghosts

TABLE I. Protein Amounts Extracted During the Isolation of Muscle Glycoprotein*

	Protein (mg)	Percent total extracted protein
1st 4 M urea supernatant	2,800	83
2nd 4 M urea supernatant	350	10
1st 8 M urea supernatant	180	5
2nd 8 M urea supernatant	16	0.5
8 M urea/50 mM mercaptoethanol 8,000g supernatant	15	0.4
8 M urea/50 mM mercaptoethanol 112,000g supernatant	6.2	0.2
Preparative gel extract	0.38	0.01

*Results of a typical experiment using a single rat.

with 4 M urea removed nearly all of the actin and much of the myosin, leaving the 130,000-dalton glycoprotein as one of the major proteins that could be solubilized by SDS (Fig. 2C). In Giemsa-stained slides of 4 M urea-treated ghosts almost no nuclei or background staining remained, only empty ghosts (Fig. 1C).

When we treated muscle ghosts with 8 M urea/0.3 M 2-mercaptoethanol as described by Hudson and Spiro [17] for the solubilization of basement membranes, the major glycoprotein was eluted as a major component of the extract. Essentially none of the major glycoprotein was extracted by 8 M urea in the absence of the reducing agent. The combination of urea and mercaptoethanol did not completely solubilize the muscle ghosts; shrunken myotube fragments still remained.

Isolation of the Glycoprotein From Homogenized Muscle

Having learned how to enrich ghosts preparations for the major 130,000-dalton glycoprotein and how to elute it, we decided to isolate and characterize it. Quantities obtainable from rat muscle ghosts were inadequate for characterization, so we developed a method for isolation of the glycoprotein directly from homogenized adult rat skeletal muscle. A flow chart outlining the glycoprotein isolation procedure is given in Figure 3 (see Methods for details).

The isolation procedure begins with two preliminary extractions with 4 M urea and two with 8 M urea. The 130,000-dalton glycoprotein is then extracted from the muscle residue with 8 M urea/50 mM 2-mercaptoethanol and purified from this extract by differential centrifugation and preparative SDS PAGE. The amount of protein obtained during the isolation and SDS-PAGE profiles of various fractions are given in Table I and Figure 4. Proteins solubilized by 4 M urea appear in Coomassie blue–stained SDS gels to be nearly identical to those present in preparations of the starting material—ie, homogenized muscle (see Fig. 4-1, 4-2). Most urea-extractable protein is released in this step. Incubation with 8 M urea releases residual actomyosin (see Fig. 4-3). Numerous glycoproteins are detected in this extract, but the amount of the major glycoprotein solubilized is relatively small since it is barely detectable in Coomassie blue–stained gels. Subsequent extraction of the muscle residue with 50 mM 2-mercaptoethanol in 8 M urea solubilizes the bulk of the 130,000-dalton glycoprotein from the residue sedimented at 8,000g (Fig. 4-4, 4-5). It is the major component of the extract stained with Con A and one of the major components stained by Coomassie blue. Frequently, the glycoprotein is seen to be a very closely spaced doublet.

Isolation of Muscle Glycoprotein

Homogenized adult rat skeletal muscle
Extract 2 × with 4 M urea
Centrifuge 8,000g

→ (supernatant)

↓ (pellet)
Extract 2 × with 8 M urea
Centrifuge 8,000g

→ (supernatant)

↓ (pellet)
Extract with 8 M urea/50 mM
2-mercaptoethanol
Centrifuge 8,000g

→ (supernatant)
Centrifuge 112,000g

→ (supernatant)
Preparative gel electrophoresis
Elute glycoprotein

↓ (pellet)

↓ (pellet)

Fig. 3. Outline of the procedure for isolation of the major muscle glycoprotein. Further details are given in text under Methods.

The mercaptoethanol extract is cloudy but can be cleared by centrifugation at 112,000g. Approximately half of the total protein is pelleted by this step, but at least 90% of the glycoprotein remains in the supernatant in which it constitutes the major protein (Fig. 4-6, 4-7). Nearly all of the unidentified protein that migrates as a major band just ahead of the glycoprotein on SDS gels is pelleted by centrifugation at 112,000g, as are most of the low molecular weight proteins. We isolated the glycoprotein from the 112,000g supernatant by preparative SDS gel electrophoresis (Fig. 4-8). The glycoprotein was precisely localized in unfixed gels by differential precipitation with 4 M sodium acetate, cut out, and eluted with urea and mercaptoethanol. Glycoprotein isolated in this manner was used for amino acid analysis and to immunize animals.

Although 8 M urea with mercaptoethanol has been employed to solubilize glomerular basement membrane [17], treatment with this solvent does not appreciably reduce the size of the pellet obtained at 8,000g from muscle. Much of the protein in the pellet cannot be solubilized even by boiling with urea, SDS, and mercaptoethanol. The SDS-insoluble residue contains pieces still recognizable as muscle ghosts. The

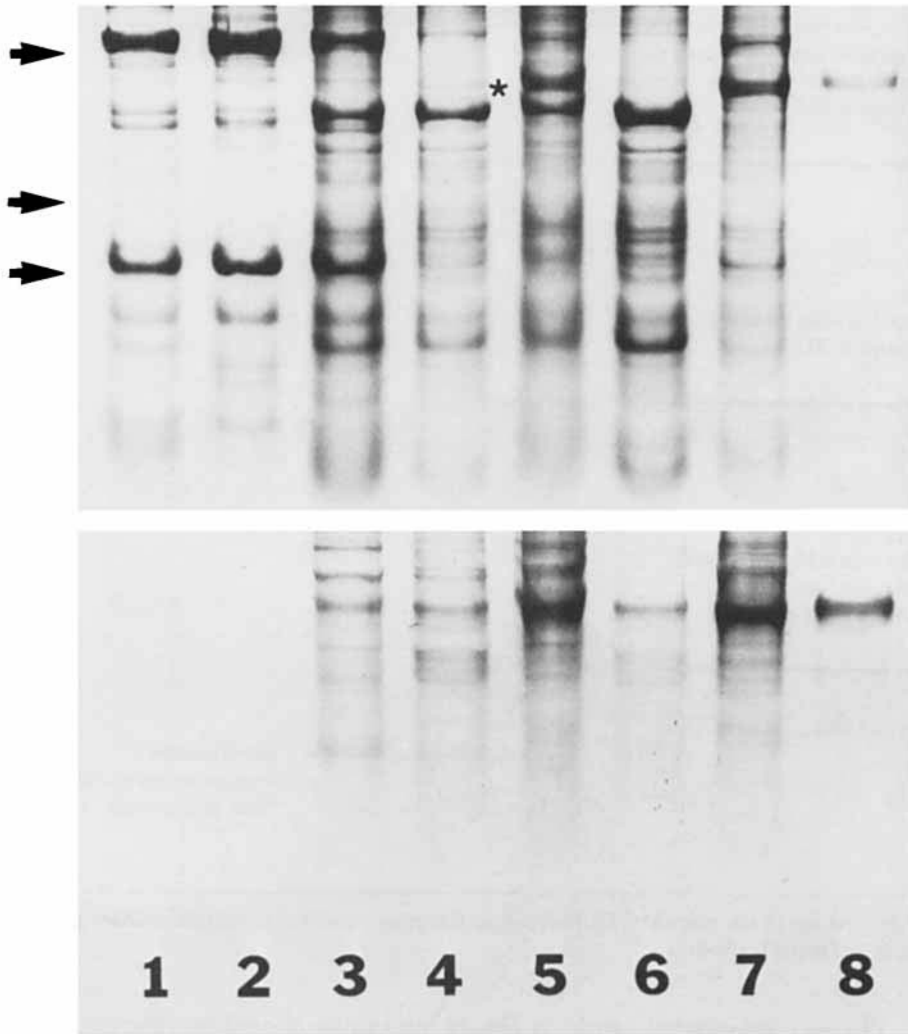

Fig. 4. SDS-PAGE of samples obtained during isolation of the major glycoprotein. The samples layered were 1) washed homogenized muscle; 2) first 4 M urea extract; 3) first 8 M urea extract; 4–7) 8 M urea/50 mM 2-mercaptoethanol extraction; 4) 8,000g pellet; 5) 8,000g supernatant; 6) 112,000g pellet from 8,000g supernatant; 7) 112,000g supernatant from 8,000g supernatant; and 8) extract from preparative gel. The upper and lower gels were run simultaneously and stained using Coomassie blue and Con A, respectively. Identical amounts of the paired samples were layered on each gel. The arrows to the left from above down indicate the mobilities of myosin, albumin, and actin in the gels. The extreme sensitivity of Con A staining is best illustrated in channel 6, where the major glycoprotein is visible in the lower lectin-treated gel whereas virtually nothing is detectable in the upper gel stained with Coomassie blue. The asterisk indicates the two major collagen bands, which become pink with prolonged destaining.

TABLE II. Amino Acid Composition: Muscle Glycoprotein vs Other Extracellular Glycoproteins (Amino Acid Residues per 1,000)

	Muscle GP	Muscle GP (DEAE)	Fibronectin	Laminin	Type III collagen	Type V (AB_2) collagen
Half-Cys	8	7	26	30	2	ND
Hyp	19	16			112	112
Asp	99	104	92	109	53	50
Thr	46	47	97	58	10	21
Ser	109	150	68	77	42	28
Glu	144	140	116	122	72	89
Pro	67	55	76	53	109	111
Gly	158	159	80	93	360	321
Ala	75	70	43	76	86	48
Val	38	33	81	48	16	31
Met	14	11	11	14	7	9
Ile	26	19	44	42	12	18
Leu	52	43	57	92	16	38
Tyr	15	16	45	27	3	2
Phe	27	22	27	31	8	13
His	17	22	21	24	8	9
Hyl	11	11		2	5	31
Lys	25	28	36	52	35	19
Arg	45	38	52	50	44	56
Trp	ND	ND	28	ND		
References			[20]	[20]	[21]	[22]

SDS-PAGE profile of this pellet (Fig. 4-4) illustrates those proteins in the residue that can be solubilized with SDS. A number of the extracted proteins are identifiable as collagens by metachromatic staining with Coomassie blue [18]. After extensive destaining of the gels with 10% acetic acid, the collagen bands become pink. The major pink bands correspond to the $\alpha 1$ and $\alpha 2$ chains of type I collagen (see Fig. 4-4). The $\alpha 1$ chain migrates near the leading edge of the major glycoprotein.

Characterization of the Glycoprotein

The results of amino acid analysis of the glycoprotein isolated by urea mercaptoethanol extraction and preparative electrophoresis are given in Table II along with reported compositions of human serum fibronectin, laminin, and types III and V collagen. These other proteins are extracellular components known to be associated with muscle cell surface or basement membrane. The amino acid composition of our glycoprotein does not correspond to that of any of the other proteins.

The high glycine content and the presence of hydroxyproline and hydroxylysine in our glycoprotein preparations could have indicated significant contamination by collagen. We therefore introduced a DEAE ion-exchange chromatography step into our glycoprotein isolation procedure to remove collagen. Centrifuged urea-mercaptoethanol muscle extract was equilibrated with DEAE-Sephadex in the presence of 2 M urea/50 mM imidazole, pH 6.5. Collagen does not bind to DEAE under these conditions [19], but nearly 90% of the protein of the extract was bound. By increasing the ionic strength with 0.4 M NaCl, half the bound protein, including the 130,000-dalton glycoprotein, was released.

An amino acid analysis of glycoprotein isolated with this additional chromatography step is also given in Table II. There was very little change in composition, especially in the levels of hydroxylysine, hydroxyproline, and glycine. This indicates that there was minimal, if any, contaminating collagen in preparations isolated without DEAE chromatography. Some contaminant was removed by this procedure, however. Electrophoresis of the material that did not bind to DEAE revealed a minor Coomassie blue–staining band at the position of the 130,000-dalton glycoprotein, but this protein did not stain with Con A, and we were not able to detect any of the major glycoprotein in this unbound fraction with a quantitative micro-ELISA.

To determine whether, instead of collagen contamination, there might be a collagen-like domain within the glycoprotein molecule, we treated muscle urea-mercaptoethanol extract with purified bacterial collagenase. Using an enzyme:substrate weight ratio of approximately 1:80, the 130,000-dalton protein was completely fragmented within 15 min at 37°C. A considerable amount of new Coomassie blue–staining protein appeared in three different lower molecular weight bands (Fig. 5A). All of the new fragments contain carbohydrate, for they all stained with Con A. The fragments were stable; there was no apparent further digestion when the collagenase incubation was extended to 2½ hr. When we treated types I, III, and V collagen with collagenase, no such fragments remained; collagen was totally digested. All three low molecular weight fragments probably are derived from the 130,000-dalton glycoprotein, as we were able to demonstrate by immunoblotting. Collagenase-treated and untreated samples were run on SDS gels, and the separated proteins were blotted onto nitrocellulose paper. The paper was then stained using an antiserum against the 130,000-dalton glycoprotein followed by a peroxidase-conjugated antirabbit immunoglobulin serum (Fig. 5B). The very intense 130,000-dalton glycoprotein band was completely abolished following collagenase digestion, and three weakly staining bands appeared that correspond in mobility to the fragments detected with Coomassie blue and Con A.

On the basis of relative mobility in the SDS gels, the apparent molecular weights of the three collagenase digestion fragments are 70,000, 55,000, and 35,000. Each appears to be distinct. The relative proportion of Con A to Coomassie blue staining is much greater for the 70,000-dalton species than for the 35,000-dalton species. In the immunoblots the smallest fragment stained with a gray cast whereas all the other bands were brown. The intermediate-sized fragment appears as a doublet on the immunoblot and possibly in the Coomassie blue–stained gels, but only a single weak band is seen in the lectin-stained gels. We cannot offer any explanation based on our present data why the sum of the apparent molecular weights of the fragments (160,000) is greater than the molecular weight of the intact glycoprotein.

Following the extremely sensitive immunoblotting procedure, there was also weak staining of additional bands in the untreated mercaptoethanol extract (Fig. 5B). Our antiserum is polyclonal, and antibody to contaminating proteins with molecular weights close to that of the major glycoprotein could be contained within it. It is difficult to evisage how proteins with molecular weights so different from that of the glycoprotein could have contaminated our antigen preparations, and we conclude that the additional proteins revealed in the immunoblots are likely to be antigenically related to the glycoprotein. There are three lower molecular weight species which are not noticeable in the Coomassie blue–stained gel and which are just barely detectable in the gel stained with Con A. They may be proteolytic fragments of the 130,000-

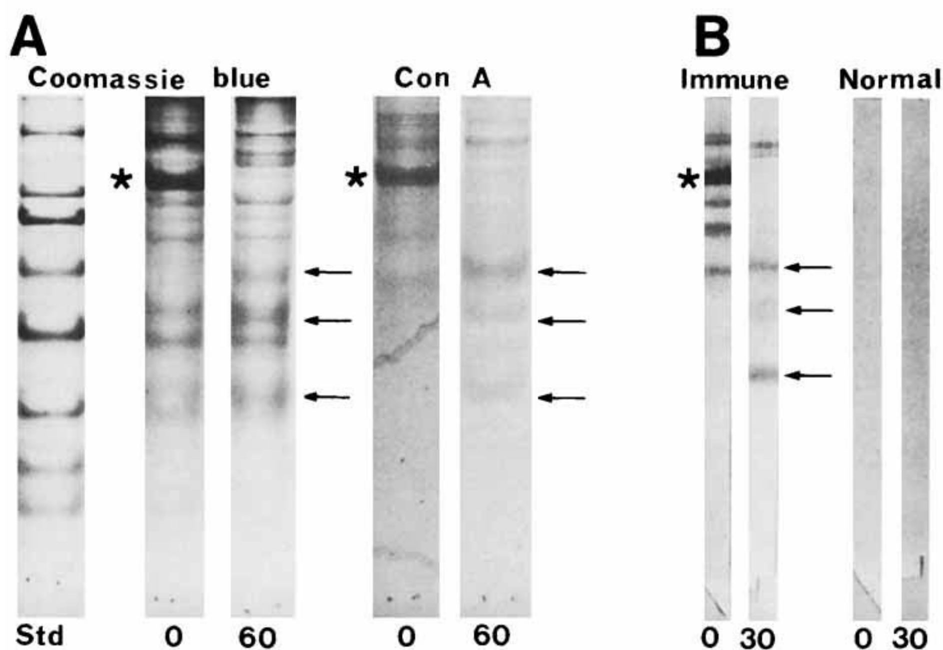

Fig. 5. SDS-PAGE and immunoblots of collagenase-digested muscle glycoprotein. Aliquots of muscle urea-mercaptoethanol extract containing 40 μg of protein were incubated at 37°C with 24 units of purified bacterial collagenase (approximate wt ratio, 80:1) for the times (min) indicated. Coomassie blue- and Con A-stained gel profiles of control and digested samples are shown in A. The 130,000-dalton glycoprotein is indicated by asterisks, and three new low molecular weight bands by arrows. The 130,000-dalton glycoprotein is completely fragmented by collagenase. All three fragments stain with Con A and Coomassie blue. Molecular weight standards from the top down are myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. A second set of collagenase digestion samples was blotted onto nitrocellulose immediately after SDS-PAGE. The blots were stained using rabbit sera prepared against the 130,000-dalton glycoprotein (immune) or adjuvant (normal) followed by peroxidase-conjugated antirabbit immunoglobulin. The results are shown in B. The 130,000-dalton glycoprotein stained very intensely with the immune serum, and bands corresponding to the three collagenase fragments detected with Coomassie blue and Con A were also stained. There was no staining with control serum.

dalton glycoprotein. None of these bands is present in the blot of the collagenase-digested extract. More retarded in the SDS gels is a very high molecular weight glycoprotein complex which would also appear to be antigenically cross-reactive with the predominant 130,000-dalton species. The relationship of these high molecular weight glycoproteins to the major band is not clear. Their mobility increases only slightly following collagenase treatment.

By immunochemistry we have found the 130,000-dalton glycoprotein to be distinct from types I, III, IV, and V collagen, and from fibronectin and laminin. In view of the fact that the glycoprotein apparently has a collagenous domain and that the glycoprotein and types I, III, and V collagens migrate at similar positions on SDS-PAGE, we were concerned whether the antisera we raised in rabbits immunized with glycoprotein isolated by preparative electrophoresis would react with collagen. We tested the antisera for antibodies to types I, III, and V collagen by hemagglutination. The results of a typical test are given in Table III. A second antiglycoprotein serum gave similar titers. Neither serum showed any reaction with any of the collagens.

TABLE III. Agglutination Titers ($-\log_2$) for Muscle Glycoprotein and Collagen of Rabbit Sera Against Isolated Glycoprotein and Type III Collagen

	Cell coating			
Serum	Muscle GP	Type I collagen	Type III collagen	Type V collagen
Normal rabbit	1	2	1	2
Antimuscle GP	8	1	1	1
Anti-type-III collagen	1	1	11	1

TABLE IV. Micro-ELISA Comparison of Muscle Glycoprotein and Other Extracellular Matrix Proteins (absorbance 450 nm)

	Sera (dilution)			
Antigen	Antimuscle GP (10^{-4})	Antifibronectin (10^{-5})	Antilaminin (10^{-4})	Anti-type-III collagen (10^{-3})
Muscle GP	1.11; 1.09	0.04; 0.05	0.01; 0.00	0.07; 0.07
Fibronectin	0.02; 0.00	>1.8 ;>1.8	0.01; 0.00	0.03; 0.04
Laminin	0.01; 0.00	0.03; 0.02	1.43; 1.17	0.02; 0.02
Type I collagen	0.02; 0.01	0.03; 0.02	0.01; 0.01	0.26; 0.24
Type III collagen	0.02; 0.00	0.01; 0.01	0.00; 0.00	1.49; 1.48
Type IV collagen	0.02; 0.00	0.01; 0.00	0.01; 0.00	0.04; 0.04
Type V collagen	0.02; 0.00	0.01; 0.01	0.01; 0.01	0.44; 0.43

We confirmed and extended these findings by means of micro-ELISA assays to include the basement membrane-associated proteins, type IV collagen, fibronectin, and laminin. Microtiter wells were coated with the different proteins; rabbit antisera against the muscle glycoprotein, fibronectin, laminin, and type III collagen were introduced; and then immunoglobulins that bound to proteins in the wells were detected with a peroxidase-conjugated second antibody and orthophenyldiamine.

Table IV presents absorbance data from an experiment in which the above designated proteins were studied with a battery of antisera. Muscle glycoprotein in this case was isolated without DEAE chromatography. Antimuscle glycoprotein serum at a dilution of 10^{-4} showed absolutely no reaction with any of the other proteins but gave a strong reaction with the immunogen. Similarly, the antifibronectin and antilaminin sera bound to their corresponding immunogen and not to the other proteins, including the 130,000-dalton muscle glycoprotein. The anti-type-III collagen serum bound most strongly to type III collagen, but there was also weak binding to types I and V. The values for antifibronectin, laminin, and collagen sera binding to the muscle glycoprotein are less than values we observed with a control rabbit serum.

We also used the antisera we prepared against the 130,000-dalton glycoprotein to determine its localization in muscle sections by PAP immunohistochemistry (Fig. 6). As expected, the glycoprotein appeared to be associated with muscle cell surfaces, for there was heavy staining in a layer surrounding each muscle cell. In some areas, the staining branched out into the regions between muscle cells, but no noticeable staining of perimysial connective tissue was found. Major blood vessels also stained. Control slides treated with serum from adjuvant immunized rabbits showed only pale staining within myotubes, and none at the cell surfaces.

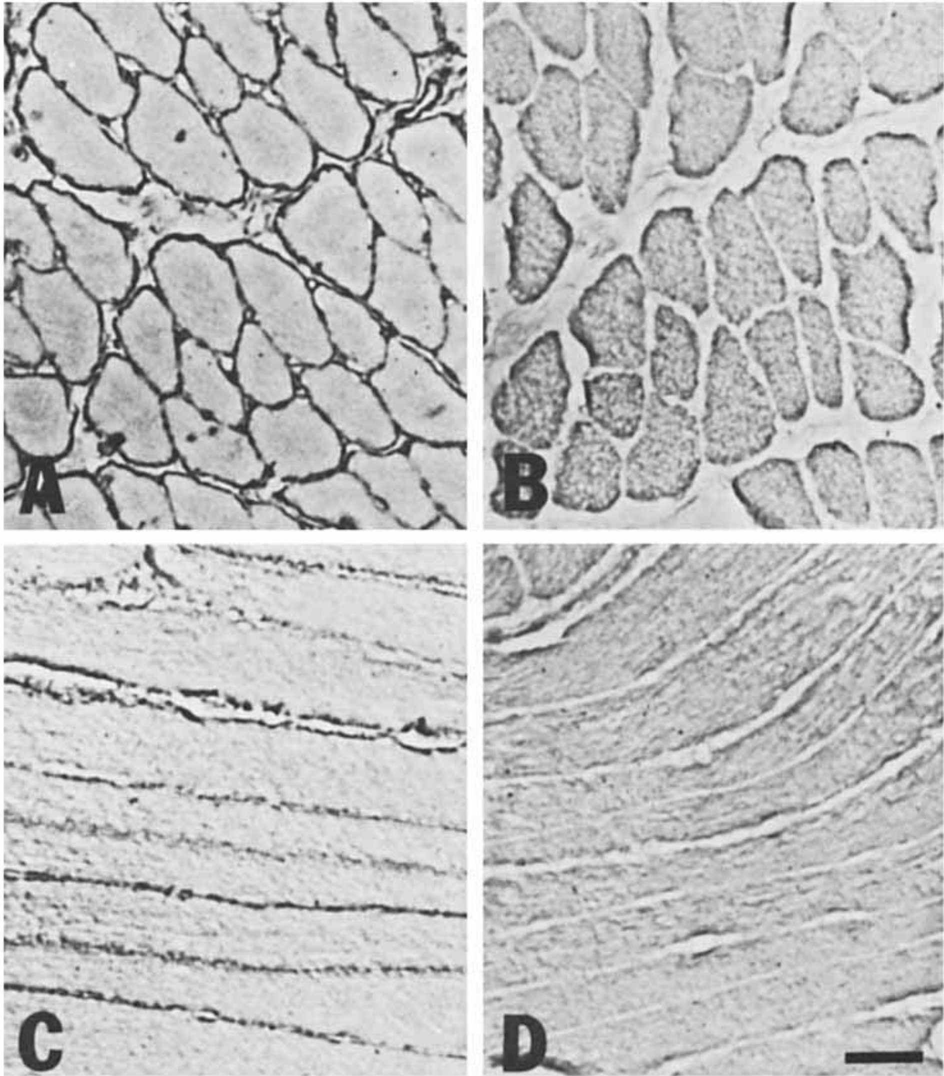

Fig. 6. PAP immunohistochemical staining of muscle with antiglycoprotein serum. Cross (A, B) and longitudinal (C, D) sections of fresh frozen rat skeletal muscle were stained with either antiglycoprotein serum (A, C) or serum from an adjuvant-immunized rabbit (B, D) according to the procedure of Sternberger [15]. Sections treated with antiglycoprotein serum stain primarily at the muscle cell surfaces. Very little stain is present in the perimysial regions. Myotubes in the control slides were not outlined with peroxidase stain. The intracellular contents appear darker in the control sections because of the necessity to increase contrast for photography. (Bar represents 50 μm.)

Staining was not restricted to muscle. Antibodies bound to sections of kidney, brain, liver, and small intestine (Fig. 7). The antiglycoprotein antibody outlined both tubules and glomeruli in the kidney and stained the capillary tufts within Bowman's capsule (Fig. 7A). Only blood vessels stained in brain (Fig. 7B). There was very fine reticulin-like staining of liver sinusoids in addition to blood vessels (Fig. 7C). In

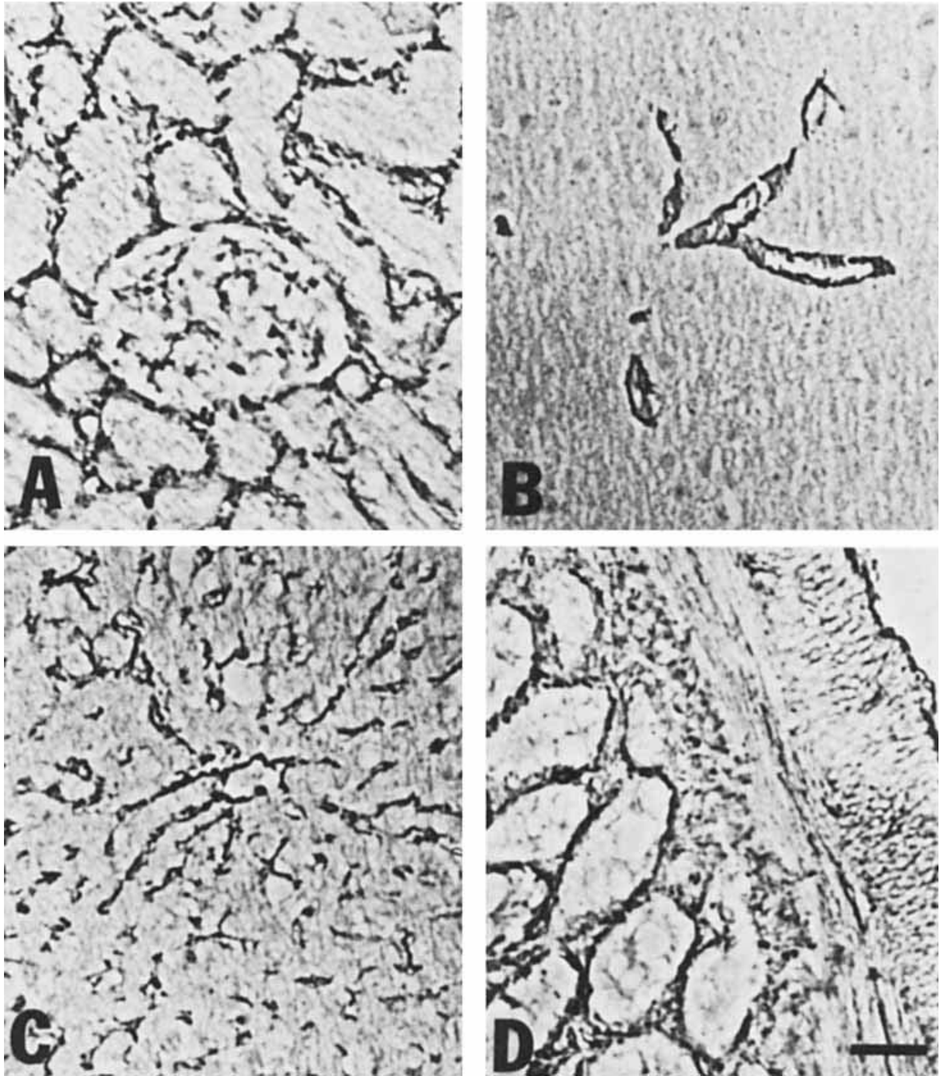

Fig. 7. PAP immunohistochemical staining of nonmuscle tissues with antiglycoprotein serum. Fresh frozen sections of rat (A) kidney, (B) brain, (C) liver, and (D) small intestine bound antibodies from our antiglycoprotein sera in very specific patterns, whereas there was no detectable binding with sera from control adjuvant-immunized rabbits. The antibody staining is almost reticulin-like. Note the tubular, glomerular, and intraglomerular staining of kidney; that blood vessels stain in brain; sinusoids are outlined in liver; and epithelial and smooth muscle basement membranes stain in small intestine (Bar represents 50 μm.)

small intestine there was a heavy network of stain at the base of the epithelial cells and within the lamina propria stained fibers outlined capillaries (Fig. 6D). Circular and longitudinal smooth muscle cells were delineated in the outer layers of the intestine. In sections of rat sciatic nerve there was staining of endoneurium and blood vessels that was similar to the pattern of staining in muscle. There was no staining of any of the tissues with control serum.

DISCUSSION

We had set out to identify and characterize the proteins associated with adult skeletal muscle cell surfaces. Despite the fact that we found published muscle ghost isolation procedures [9, 10, 23] to be unsatisfactory for our purpose, we were able to identify a major 130,000-dalton glycoprotein that was enriched in these imperfect preparations and which seemed to be associated with basement membrane. It was unlikely that the glycoprotein was a plasma membrane component, for it remained in ghost preparations treated with 3% Triton X-100; electron microscopy had shown previously that 1% Triton X-100 removes all detectable plasma membrane from the muscle ghosts and leaves basement membrane as the primary visible structure [24]. We proceeded to isolate the glycoprotein and prepare antisera. By immunohistochemistry we have found the distribution of the glycoprotein in skeletal muscle sections to coincide with the localization of basement membranes.

The 130,000-dalton glycoprotein is probably not a uniquely "muscle" basement membrane component. All tissues we have checked thus far (ie, kidney, gut, liver, brain, and peripheral nerve) contain protein that is immunologically cross-reactive with the muscle glycoprotein and cross-reactive material is confined to regions containing basement membrane. The pattern of immunoperoxidase staining is reticulin-like, but not in the fine linear distribution that would be expected of a basal lamina component. Sinusoidal staining is detected in liver; apparent mesangial staining is seen in glomeruli (Fig. 7). These localizations are similar to that of fibronectin. In muscle the distribution of our glycoprotein approximates that of type V collagen. It is not restricted as exclusively to the cell surface as is type IV collagen [25], nor is it as abundant as types I and III collagen in the perimysium [4, 26].

Many properties of the 130,000-dalton glycoprotein distinguish it from other known reticulin or basement membrane proteins. Its lower molecular weight immediately distinguishes it from laminin and fibronectin. Unlike intact laminin or collagen, the glycoprotein binds tightly to DEAE in the presence of 2 M urea [19, 20]. The amino acid composition of the glycoprotein indicates that it is not a collagen per se. The high serine and glycine contents and the presence of hydroxylysine distinguish it from laminin, fibronectin, entactin [27], and a previously described noncollagen reticulin component [28, 29]. Immunochemically, we have shown that the muscle glycoprotein is not related to laminin, fibronectin or to types I, III, IV, or V collagens.

The feature that best differentiates this glycoprotein from other extracellular matrix components is the presence of collagenous domains. Because three apparently distinct glycoprotein fragments are present following collagenase digestion, we would suggest that there are at least two collagenous regions in the glycoprotein. Collagenase treatment, as employed in prior attempts to solubilize and characterize noncollagenous extracellular matrix and basement membrane components, would have digested any protein corresponding to the 130,000-dalton muscle glycoprotein, and this may have precluded its earlier recognition as a matrix component. The presence of short collagen-like regions in proteins other than collagen teleopeptides is unusual but not without precedent. Examples include complement component Clq [30], acetylcholinesterase [31], and a lung lavage glycoprotein [32].

The combination of a high concentration of urea and a reducing agent has, in addition to its use for the solubilization of basement membrane components, as in the present instance, also been used to solubilize a group of connective tissue proteins

referred to as structural glycoproteins [reviewed by Anderson, 33]. In fact, structural glycoproteins have been identified operationally as those connective tissue proteins, other than collagen, that can be removed from tissue only with a denaturant, generally in conjunction with a reducing agent. Antibodies raised against preparations of structural glycoproteins bind to basement membranes. The amino acid compositions of structural glycoproteins are not distinctive, but vary depending upon the tissue of origin [34]. It is likely that these early glycoprotein preparations included material related to the glycoprotein we have isolated from skeletal muscle.

Basement membranes are composed of collagen and noncollagenous glycoproteins, but technical problems have impeded the characterization of these molecules and, in particular, the noncollagenous glycoproteins. A primary difficulty has been the isolation of basement membrane free of contaminating cell debris and serum proteins. Further problems relate to the fact that basement membranes are highly cross-linked and the proteins are poorly soluble. Significant progress in the characterization of both collagenous and noncollagenous glycoproteins followed the discovery that certain tumors and cell lines secrete extracellular matrix components normally associated with basement membranes. Laminin and, more recently, entactin were discovered in this way. In marked contrast, we have isolated a basement membrane-associated protein not from isolated membranes or from tumor matrices, but directly from homogenized tissue.

The only previous biochemical characterization of muscle basement membrane components was that of Zacks et al [10, 35]. "External lamina substance" was extracted from muscle ghosts with lithium 3,5-diiodosalicylate. It was alleged to contain those polyanionic components of muscle basement membrane that stain with ruthenium red. The amino acid composition and the SDS gel profile suggest to us that actomyosin was a major component of this extract.

Laminin and entactin have been found by immunoelectron microscopy to be associated primarily with cell surfaces immediately adjacent to basal laminae [27, 36]. In accord with its observed localization, other studies have shown that laminin mediates the attachment of epithelial and endothelial cells to type IV collagen [37]. Immunoelectron microscopy will be necessary to determine the exact location of the 130,000-dalton glycoprotein relative to basal laminae and cell surfaces. The precise location of the glycoprotein may provide a clue to its role in extracellular matrices.

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