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A Novel Muscle Protein Located inside the Terminal Cisternae of the Sarcoplasmic Reticulum*

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An immunofluorescence study of adult rat muscle tissues with a polyclonal antibody against the RGD-directed fibronectin receptor of Friend's erythroleukemia cells ($\alpha_5\beta_1$-integrin) unexpectedly revealed a pattern of intracellular antigen distribution. Western blotting analysis of rat and rabbit membrane fractions indicated that the antibody recognizes a 167-kDa protein expressed both in heart and in skeletal muscle (relative abundance: heart > slow muscle > fast muscle), but not in liver and kidney. The 167-kDa protein did not show altered electrophoretic mobility upon reduction and failed to bind several lectins, including wheat germ agglutinin. A study of its subcellular distribution in rabbit skeletal muscle revealed that the 167-kDa protein is mostly associated with the terminal cisternae of the sarcoplasmic reticulum (SR) and, to a smaller extent, with the sarcolemma, while it is absent in the longitudinal tubules of the SR. The 167-kDa protein is not an integral membrane protein since it can be extracted at pH 10. This protein can be proteolytically cleaved only in the presence of detergent, indicating that it resides on the luminal side of the SR. The 167-kDa protein could be resolved from the closely spaced sarcalumenin and histidine-rich protein by column chromatography followed by detergent dialysis and two-dimensional gel electrophoresis. The N terminus and the internal sequences did not match any known sequence in protein and DNA data bases, indicating that the 167-kDa protein is a novel muscle protein selectively localized to the SR. Integrins from rat kidney fibroblasts were not recognized by either (i) a polyclonal antiserum against the purified 167-kDa protein or (ii) the anti-$\alpha_5\beta_1$-integrin antiserum after affinity purification onto the 167-kDa protein. These data indicate that the 167-kDa protein is not immunologically cross-reactive with integrins, despite its reaction with a polyclonal anti-integrin antibody.

Skeletal muscle is a specialized tissue converting chemical energy into mechanical work. For this reason, both the endo- and exosarcomeric compartments are equipped with a very well developed cytoskeleton, ensuring structural stability to the sarcomeres and to the myofibrils (1). In addition, skeletal muscle is equipped with a complex meshwork of skeletal muscle proteins, ensuring mechanical stability to the sarcolemma. Mechanical stress of membrane cytoskeletal actin filaments can be discharged to the extracellular matrix in at least two ways: the first is represented by the α- and β-dystroglycan complex, which binds dystrophin and actin-binding protein at the cytoplasmic side of the sarcolemma and merosin at the extracellular matrix side; the second is represented by vinculin (2), talin, spectrin, and ankyrin localized to the costameres (3, 4).

In recent years, it has become clear that integrins play a major role in mediating interactions of muscle cells with the extracellular matrix and in muscle differentiation. Integrin receptors are heterodimeric transmembrane glycoproteins composed of α- and β-subunits. Several isoforms of both α- and β-subunits have been characterized that are apparently expressed at different times in the process of muscle development (5).

Studies of the β_1-subunit revealed that it is localized to the myotendinous and neuromuscular junctions and to the costameres (6, 7). The importance of the structural role of β_1-integrin was demonstrated in studies of *Drosophila* mutants lacking β_1-subunit expression: the mutant showed an abnormal muscle tissue morphology, without proper organization of myofibers and the myotendineus junction (8).

Several integrins of the β_1-subfamily have now been shown to play a role in muscle physiology during development, including $\alpha_4\beta_1$ and its counter-receptor vascular cell adhesion molecule-1 (9, 10), $\alpha_5\beta_1$ (11, 12), $\alpha_7\beta_1$ (13, 14), and $\alpha_9\beta_1$ (15). In particular, the $\alpha_5\beta_1$-integrin is involved in the adhesion of muscle to the extracellular matrix and has been shown to colocalize with dystrophin in the adhesion plaque-like structures of myoblasts and myotubes (16). This integrin connects the extracellular matrix to the cytoskeleton because it binds fibronectin outside the cell and talin and α-actinin inside the cell. It is also intriguing that integrin recognition motifs are also present in several intracellular muscle proteins, including the N terminus REDV sequence of dystrophin (17).

To address the role of β_1-integrins in muscle physiology, we carried out a screening of adult rat muscle tissues with a polyclonal antibody against the RGD-directed fibronectin receptor of Friend's erythroleukemia cells ($\alpha_5\beta_1$-integrin). Unexpectedly, we detected a pattern of intracellular antigen distribution. Western blotting analysis indicated that the antibody recognized a 167-kDa protein expressed both in heart and in skeletal muscle, but not in liver and kidney. The 167-kDa protein is a peripheral membrane protein mostly associated with the luminal side of the terminal cisternae of the sarcoplasmic reticulum (SR)[1] and, to a smaller extent, with the

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1 propanesulfonate; IDA, iminodiacetic acid; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

sarcolemma, while it is absent in the longitudinal tubules of the SR. The 167-kDa protein could be resolved from the closely spaced sarcalumenin and histidine-rich protein by column chromatography followed by detergent dialysis and two-dimensional gel electrophoresis. Partial protein sequences revealed a unique sequence that is not present in protein or DNA data bases, indicating that the 167-kDa protein is a novel muscle protein selectively localized to the SR. Indeed, rat fibroblast integrins were not recognized by either (i) a polyclonal antiserum against the peptide *X*-Leu-Gln-Val-Thr-Trp-Arg-Ile-Thr-Asn (the inner sequence of the 167-kDa protein) or (ii) the anti-$\alpha_5\beta_1$-integrin antiserum after affinity purification onto the 167-kDa protein.

EXPERIMENTAL PROCEDURES

This study was carried out on male rats (albino Wistar) and rabbits (New Zealand) fed *ad libitum* with standard laboratory food and tap water.

Antibodies—A rabbit polyclonal antibody against the RGD-directed fibronectin receptor of Friend's murine erythroleukemia cells ($\alpha_5\beta_1$-integrin) was prepared as described (18). An IgG fraction of the immune serum was prepared by protein G-Sepharose affinity chromatography (Pharmacia Biotech Inc.) and used in most of the experiments described in this study, as specified in the figure legends. This antibody has features similar to those of a previously described antibody (data not shown, but see Ref. 18).

Antibodies reactive with the 167-kDa protein were purified from the anti-$\alpha_5\beta_1$-integrin antiserum by absorption onto immobilized 167-kDa protein obtained from two-dimensional gel separation and transfer onto nitrocellulose. The nitrocellulose strips containing the 167-kDa protein were incubated overnight at 4 °C with the anti-$\alpha_5\beta_1$-integrin antiserum diluted 1:100 in Tris-buffered saline (0.15 M NaCl and 20 mM Tris-HCl, pH 7.5). The nitrocellulose was washed three times with 0.15 M NaCl, once with 0.5 M NaCl and 0.1% Tween 20, and again three times with 0.15 M NaCl (5 min/wash). Antibodies reactive with the 167-kDa protein were detached by treatment with 0.2 M glycine, pH 2.8, for 30 s, and neutralized with 2 M Tris-HCl, pH 8, to a final pH of 7.4 after transfer to a fresh test tube.

A polyclonal antiserum against the peptide *X*-Leu-Gln-Val-Thr-Trp-Arg-Ile-Thr-Asn (Chiron Mimotopes Pty. Ltd.) (a 167-kDa protein internal sequence) was raised in New Zeland White rabbits by subcutaneous injections. For the first injection, we used 330 μg of peptide solubilized in PBS (0.15 M NaCl, 0.1 M Na_2HPO_4, and 0.1 M NaH_2PO_4, pH 7.2) and mixed 1:1 (v/v) with Freund's complete adjuvant. Rabbits were boosted twice at ~4-week intervals and six times at ~2-week intervals with 330 μg of the peptide mixed 2:1 (v/v) with Freund's incomplete adjuvant.

Immunofluorescence Staining—Samples of soleus muscles were prepared from 3–4-month-old Wistar rats. Frozen sections (8–10 μm thick) were allowed to attach to glass slides treated with 2% gelatin in PBS. After saturation with preimmune serum for 30 min, the sections were incubated for 30 min with the antibody (50 μg/ml in PBS), washed thoroughly in PBS, and reacted with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Sigma) diluted 1:10,000 in PBS. All incubations were carried out at room temperature.

Membrane Preparations—A membrane fraction from rat or rabbit fast-twitch muscle, kidney, and liver was prepared as follows. The minced tissues were passed in a Potter-Elvehjem homogenizer with a tight-fitting, rotating Teflon pestle in 0.3 M sucrose, 5 mM imidazole, and 5 mM sodium EGTA, pH 7.0 (~4 ml of solution were used per g of tissue). The homogenates were centrifuged at 6500 × g_{max} for 20 min in a refrigerated Sorvall RC5B centrifuge. The pellets were discarded, and the supernatants were centrifuged at 15,000 × g_{max} for 30 min. The supernatants were spun at 150,000 × g_{max} for 60 min in a Beckman L70 ultracentrifuge. The resulting membrane pellets were resuspended in isolation buffer at ~5 mg/ml, frozen in liquid nitrogen, and stored at −80 °C.

Terminal cisternae from rabbit fast-twitch muscle sarcoplasmic reticulum were prepared by the following method. Briefly, 200–220 g of rabbit back and hind leg muscles were homogenized in 900 ml of 0.3 M sucrose and 5 mM imidazole, pH 7.4, in a Waring blender for 2 min. The homogenate was centrifuged at 7700 × g_{max} for 20 min. The supernatant was discarded, and the homogenization and centrifugation steps were repeated for the pellet. The resulting supernatant was filtered through three layers of cheesecloth and centrifuged at 110,000 × g for 90 min in the Beckman ultracentrifuge. The pellet was resuspended in 30 ml of 2 M sucrose and 5 mM imidazole, pH 7.4; placed at the bottom of Ti-60 centrifuge tubes; and overlaid with a discontinuous sucrose gradient consisting of 1.32, 1.11, 0.90, and 0.79 M sucrose in 5 mM imidazole, pH 7.4 (5 ml/step). After centrifugation at 150,000 × g for 90 min, the membranes banding at each interface were collected; diluted in 5 mM imidazole, pH 7.4; and centrifuged at 150,000 × g for 45 min. Each pellet was resuspended in 0.3 M sucrose and 5 mM imidazole, pH 7.4; frozen in liquid nitrogen; and stored at −80 °C. The 0.79–0.90 M sucrose interface is enriched in T-tubules; the 0.90–1.11 M sucrose interface is enriched in longitudinal reticulum; the 1.11–1.32 M sucrose interface contains a mixture of longitudinal reticulum and terminal cisternae; and the 1.32–2 M sucrose interface is highly enriched in terminal cisternae of the sarcoplasmic reticulum. Triads were purified exactly as described (19); resuspended in 0.3 M sucrose and 5 mM Hepes, pH 7.1; frozen in liquid nitrogen; and stored at −80 °C. The isolation of the sarcolemma was carried out as described by Luise *et al.* (20). All buffers contained 0.23 mM PMSF, 0.83 mM benzamidine, 1 mM iodoacetamide, 1 mM leupeptin, 0.7 mM pepstatin, and 76.8 nM aprotinin.

For some experiments, a fraction enriched in the 167-kDa protein was prepared as follows. Rabbit slow-twitch (soleus) muscles (~10 g) were homogenized using a Teflon-glass homogenizer in 50 ml of 20 mM NaH_2PO_4, 20 mM $Na_4P_2O_7$, 0.3 M KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, and 1 mM EGTA, pH 7.0, for 20 s. The homogenate was centrifuged at 16,300 × g for 20 min. The supernatant was discarded, and the pellet was homogenized in 40 ml of 5 mM histidine and 0.1 mM EGTA. The homogenate was centrifuged at 40,000 × g for 30 min. The supernatant was discarded, and the pellet was resuspended in 0.6 M KCl, 5 mM histidine, 0.3 M sucrose, and 0.1 M EGTA and then centrifuged at 40,000 × g for 30 min. The final supernatant was frozen in liquid nitrogen and stored at −80 °C.

Purification of the 167-kDa Protein—Terminal cisternae membrane fractions from rabbit fast-twitch skeletal muscle were prepared as described above. Approximately 200 mg of terminal cisterna membranes in 20 ml of 0.3 M sucrose and 5 mM imidazole, pH 7.4, were diluted in 250 ml of 0.6 M NaCl, 20 mM Na_2HPO_4, and 20 mM NaH_2PO_4, pH 7.4, and gently stirred at 4 °C for 30 min. The suspension was then centrifuged at 150,000 × g_{max} for 30 min. The resulting pellet, which was largely depleted of myosin, was resuspended in 20 ml of 20 mM Na_2HPO_4 and 20 mM NaH_2PO_4, pH 7.4. After bringing the pH to 11.0 with 1 N NaOH, the suspension was gently stirred for 30 min at 4 °C and ultracentrifuged as described above. The supernatant, which contained all of the 167-kDa protein, was brought to pH 7.4 with 1 N HCl, made 0.5 M in NaCl and 0.5% (w/v) in CHAPS, and further fractionated by chromatography on IDA-Zn^{2+}-agarose (Sigma) as described below.

Ten milliliters of IDA-Zn^{2+}-agarose matrix were equilibrated with 10 volumes of acetate buffer (0.1 M sodium acetate, pH 4.5) followed by 5 volumes of 10 mM $ZnCl_2$ in acetate buffer and by at least 10 volumes of acetate buffer to remove the excess zinc. After equilibration of the column with at least 20 volumes of binding buffer (0.5 M NaCl, 20 mM Na_2HPO_4, 20 mM NaH_2PO_4, and 0.5% (w/v) CHAPS, pH 7.4), the neutralized supernatant from the last centrifugation step (see above) was loaded onto the column, and the flow-through fraction was reapplied for five times. The flow-through fraction, which contained most of the 167-kDa protein, was dialyzed against 100 mM NaCl, 10 mM Tris, pH 8.5, 2 mM dithiothreitol, and 0.1 mM PMSF. A visible precipitate formed, which was collected by centrifugation and which contained the 167-kDa protein virtually free of sarcalumenin (see Fig. 4).

SDS-PAGE and Western Blotting—SDS-PAGE (5–10% acrylamide continuous gradient gel, 50 μg of protein/lane) was carried out as described (23). The gels were stained with either Coomassie Brilliant Blue or Stains-all (Sigma) as described (24). For Western blotting, proteins were transferred to nitrocellulose sheets (Schleicher & Schuell, Dassel, Federal Republic of Germany) for 4 h at 1 A with a Hoefer apparatus at 4 °C (28). The nitrocellulose was stained with Ponceau Red (0.2%, w/v) in 3% (v/v) trichloroacetic acid, photographed, destained in H_2O, and finally blocked for 1 h at room temperature in Tris-buffered saline containing 10% (v/v) defatted milk (blocking buffer). The blocking solution was discarded, and the nitrocellulose was incubated with (i) 10 μg/ml purified immune IgG, (ii) a 1:5 dilution of the polyclonal antiserum against the 167-kDa peptide, (iii) the anti-$\alpha_5\beta_1$-integrin antiserum after affinity purification onto the 167-kDa protein (see above), or (iv) a 1:100 dilution of anti-$\alpha_5\beta_1$-integrin or irrelevant rabbit antiserum (see figure legends for details). The secondary antibody used was a goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) or with peroxidase (Calbiochem) at the recommended dilution (1:20,000 and 1:5000, respectively) in blocking buffer. After removing the antibody solution, the blots were rinsed four times with Tris-buffered saline and 0.05% Tween 20 and finally developed with nitro blue tetrazolium

or with Chemiluminescence (Boehringer Mannheim).

Two-dimensional Gel Electrophoresis—Electrofocusing was carried out on 6% polyacrylamide capillary gels containing 2% Ampholine (Pharmacia Biotech AB; pH interval of 3.5–10). The second dimension was SDS-PAGE on 5–10% acrylamide continuous gradient slab gels.

Sequence Analysis—The proteins were electroblotted onto polyvinylidene difluoride membrane in a semidry apparatus (Hoefer) using a Towbin (28) buffer (50 mM Tris-HCl, 192 mM glycine, 0.02% (w/v) SDS, 10% (v/v) methanol, and 2 mM dithiothreitol) for 1 h at ~1.2 mA/cm^2. The transferred protein was visualized on the membrane by a 5-min incubation in 0.1% (w/v) Serva blue R in 50% (v/v) methanol, followed by destaining in 70% (v/v) methanol for 5–10 min. The dried polyvinylidene difluoride membrane slices were cut into small pieces (~1 mm^2) and equilibrated for 1 h at room temperature in 10 μl of 1% (w/v) β-octyl glucopyranoside and 100 mM ammonium bicarbonate, pH 7.8. Digestion was initiated by the addition of 1 μg of trypsin (Promega) in 1 μl of the same buffer and was carried out for 15 h at room temperature. Digestion was stopped by the addition 1 μl of 2% (v/v) trifluoroacetic acid to the sample. The supernatant was collected; membrane pieces were washed twice with 10 μl of 0.1% trifluoroacetic acid; and each wash was pooled with the supernatant. The peptide mixture was resolved by reverse-phase high pressure liquid chromatography on a capillary column (C_{18}, 5 μm, 300 Å, 280 × 0.32 mm; LC Packings International, Zürich). The total flow was 3 μl/min, and the column was washed extensively with solvent system A (0.1% (v/v) trifluoroacetic acid in H_2O) before running a 60-min linear gradient from 0 to 70% solvent system B (80% (v/v) acetonitrile and 0.08% (v/v) trifluoroacetic acid). Peptides were collected directly onto trifluoroacetic acid-treated glass fiber discs that had been precoated with Polybrene. These were analyzed on an Applied Biosystems 476A Protein Sequencer, with sequencing cycles being run according to standard protocols provided by the manufacturer.

Cell Cultures and Extraction—Normal rat kidney fibroblasts were grown to 90% confluency in Dulbecco's modified Eagle's medium (Poiesys, Padova, Italy) supplemented with 10% heat-inactivated fetal calf serum, 20 mM glutamine, and penicillin/streptomycin in a CO_2 incubator. Cells in 75-cm^2 tissue culture flasks (Greiner) were washed twice with ice-cold PBS and then extracted on ice with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris/MOPS, pH 7.4, 1 mM PMSF, and 0.2 M β-octyl glucopyranoside (2 ml of extraction buffer/flask). After 20 min, the bottoms of the flasks were carefully scraped with a rubber policeman, and the extract was transferred to polypropylene test tubes. After a further 20 min on ice, the extracts were cleared by centrifugation at 15,000 × g_{max} in an Eppendorf microcentrifuge. Samples were boiled with an equal volume of double-strength Laemmli gel sample buffer (21) under nonreducing conditions and subjected to SDS-PAGE and Western blotting as described above.

RESULTS AND DISCUSSION

To study the expression of β_1-integrins in adult rat tissues, we screened a series of frozen sections with a rabbit polyclonal antibody against the fibronectin receptor ($\alpha_5\beta_1$-integrin) of Friend's erythroleukemia cells (18). No specific staining beyond the vascular compartment was seen in liver and kidney (data not shown), while a specific reaction was revealed with soleus muscle (Fig. 1) and with heart and extensor digitorum longus muscles (data not shown). It can be seen that besides the intense reactivity of endothelial cells in the capillaries surrounding the muscle fibers, the fluorescence displayed a clear reactivity with the muscle fibers themselves (Fig. 1*B*). A prominent banding pattern with periodicity of ~1.3–1.5 μm could be observed, while no reactivity was detected with the secondary antibody alone (Fig. 1*A*). These experiments indicate that the antibody recognizes a muscle-specific antigen that appears to be expressed both in heart and in slow and fast skeletal muscle. The overall pattern of fluorescence distribution suggests, unexpectedly, that the antigen may be localized within either the T-tubules or the terminal cisternae.

To identify the protein(s) responsible for the fluorescence labeling, a Western blot analysis of total membrane fractions from rat soleus muscle was carried out. Fig. 1*C* shows that the 167-kDa protein was recognized in rat soleus muscle (*lane 1*), but not in kidney (*lane 2*). As described above, the overall pattern of antigen expression in rat muscle suggested an intracellular distribution of the 167-kDa protein. This finding is strengthened by the fact that a protein of the same size was recognized by this rabbit antibody in rabbit muscles. Indeed, a 167-kDa protein was revealed in heart (*lane 3*), gastrocnemius (*lane 4*), soleus (*lane 5*), and psoas (*lane 6*) muscles from rabbit. The reaction with this protein was specific since an irrelevant rabbit antiserum gave no detectable signal (*lanes 7–10*), while the intensely stained band at ~70 kDa was also detected by an irrelevant rabbit serum and is therefore not specific.

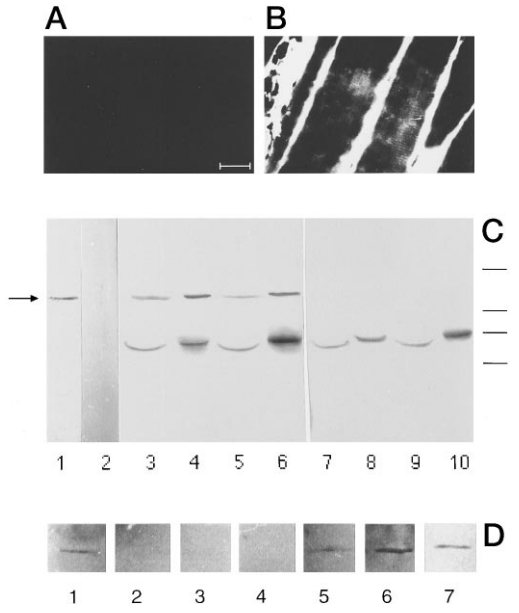

FIG. 1. **Reactivity of rat and rabbit tissues with a mouse anti-fibronectin receptor ($\alpha_5\beta_1$-integrin) antibody.** *A* and *B*, 10-μm-thick frozen sections from soleus muscle were reacted with the anti-receptor antibody followed by a secondary goat anti-rabbit IgG fluoresceinated antibody (*B*) or by the secondary antibody alone (*A*) as described under "Experimental Procedures" and visualized by fluorescence microscopy. *Bar* = 32 μm. *C*, Western blot analysis was carried out on total membrane fractions from rat soleus muscle (*lane 1*) and kidney (*lane 2*) and from rabbit heart (*lanes 3* and *7*), gastrocnemius (*lanes 4* and *8*), psoas (*lanes 5* and *9*), and soleus (*lanes 6* and *10*) muscles with the anti-receptor antibody (*lanes 1–6*) or with an irrelevant rabbit antiserum (*lanes 7–10*). Molecular mass standards (*bars*) were rabbit muscle myosin (205 kDa), *Escherichia coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), and bovine serum albumin (66 kDa). *D*, Western blot analysis was carried out on rabbit tissues with the anti-receptor antibody. *Lane 1*, skeletal muscle homogenate; *lane 2*, liver homogenate; *lane 3*, kidney homogenate; *lanes 4–7*, skeletal muscle subcellular fractions: cytosol, total membranes, sarcolemma, and terminal cisternae, respectively.

Fig. 1*D* illustrates the pattern of distribution of the 167-kDa protein in rabbit tissues. The protein was detected in a total homogenate from skeletal muscle (*lane 1*), but not from liver (*lane 2*) or kidney (*lane 3*). The subcellular distribution of the 167-kDa protein in skeletal muscle was investigated further. It can be seen that the protein was not present in a cytosolic fraction (*lane 4*), while it was clearly detected in a total membrane fraction (*lane 5*), in purified sarcolemma (*lane 6*), and in the terminal cisternae of the SR (*lane 7*). This experiment clearly indicates that the 167-kDa protein is membrane-associated.

To investigate the nature of the interactions of the 167-kDa protein with muscle membranes, a fraction enriched in terminal cisternae was exposed to increasing pH. The solubilized proteins and the membranes were then separated by either ultracentrifugation or filtration through 0.45-μm pore-sized filters, and each fraction was separated by SDS-PAGE and probed with the anti-receptor antibody after transfer to nitrocellulose filters. Fig. 2 shows the Western blot of such an

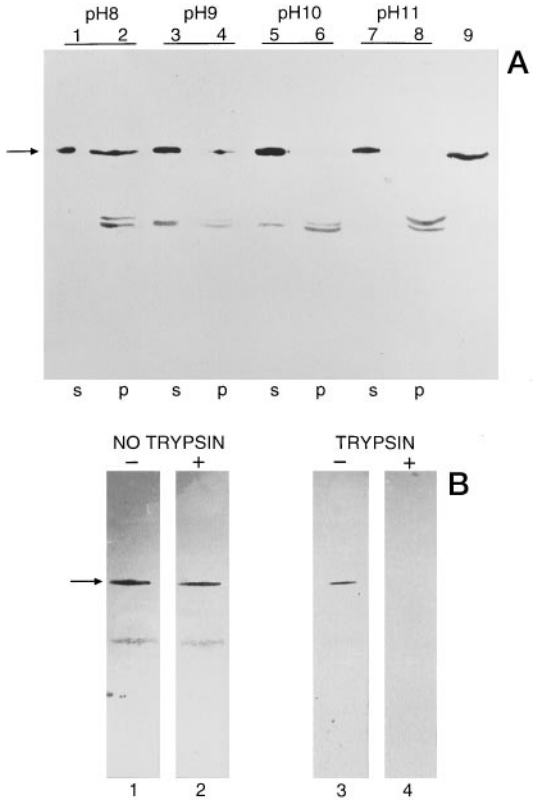

FIG. 2. **Extraction of the 167-kDa protein at alkaline pH and trypsin sensitivity in the presence of detergent.** *A*, membrane fractions enriched in terminal cisternae from rabbit fast-twitch skeletal muscle were incubated in 0.3 M sucrose, 5 mM imidazole, 5 mM EDTA, 5 mM EGTA, and NaOH to give a final pH of 8, 9, 10, or 11. After incubation for 30 min at 0 °C with constant stirring, a soluble and a membrane fraction were prepared by centrifugation at 200,000 × g_{max} for 60 min. Each fraction was separated by SDS-PAGE, transferred to nitrocellulose, and probed with the anti-receptor antibody. *s*, supernatant; *p*, pellet. *Lanes 1* and *2*, pH 8; *lanes 3* and *4*, pH 9; *lanes 5* and *6*, pH 10; *lanes 7* and *8*, pH 11; *lane 9*, untreated SR terminal cisternae fraction. *B*, duplicate aliquots of the same SR terminal cisternae fraction were incubated with (*lanes 3* and *4*) or without (*lanes 1* and *2*) trypsin (80:1 ratio to trypsin in weight) in 0.3 M sucrose and 5 mM imidazole, pH 7.4, in the presence (+; *lanes 2* and *4*) or absence (−; *lanes 1* and *3*) of 1% (v/v) Triton X-100. After 3 min at room temperature, all reactions were transferred on ice, and soybean trypsin inhibitor (2:1 ratio to trypsin) was added, immediately followed by solubilization in SDS and separation by SDS-PAGE. After transfer to nitrocellulose, the proteins were stained with Ponceau Red, destained, and probed with the anti-receptor antibody.

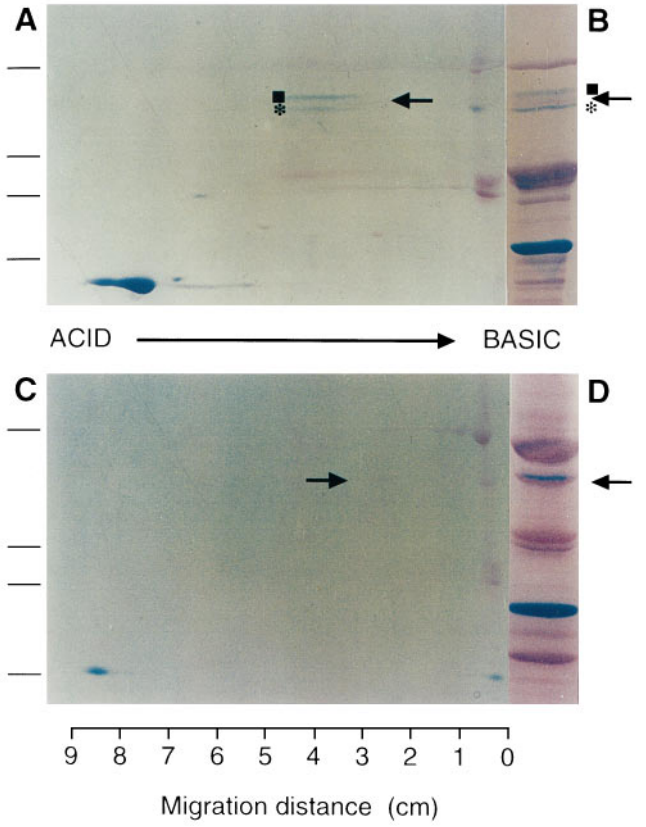

FIG. 3. **SDS-PAGE and two-dimensional separation of terminal cisterna proteins.** *A* and *B*, an SR terminal cisternae fraction was separated by SDS-PAGE (*B*) or by isoelectrofocusing followed by SDS-PAGE (*A*) and stained with Stains-all dye. Note that a non-metachromatic 167-kDa protein (←) with a slightly higher isoelectric point can be resolved from the closely spaced histidine-rich protein (■) and sarcalumenin (*). *C* and *D*, a pH 11 extract from a rabbit SR terminal cisternae fraction was neutralized and fractionated by chromatography on an IDA-Zn^{2+}-agarose column. The flow-through fraction, containing most of the 167-kDa protein and sarcalumenin, was dialyzed against 100 mM NaCl, 10 mM Tris, pH 8.5, 2 mM dithiothreitol, and 0.1 mM PMSF. A visible precipitate formed, which was collected by centrifugation and separated by SDS-PAGE (*D*) or by isoelectrofocusing followed by SDS-PAGE (*C*) and stained with Stains-all dye. Note the substantial enrichment of the non-metachromatic 167-kDa protein (←) and the absence of sarcalumenin (compare with *B*).

experiment. The 167-kDa protein was membrane-associated at pH lower than 9, was partially solubilized at pH 9, and was completely recovered in the soluble fraction at pH 10 or higher. These findings indicate that the 167-kDa protein is a peripheral membrane protein. To obtain information about its location, we subjected a terminal cisternae membrane fraction to controlled proteolysis with trypsin in the absence or presence of Triton X-100. After blocking the reaction by the addition of soybean trypsin inhibitor and boiling in SDS gel sample buffer (21), Western blotting with the anti-receptor antibody was carried out after SDS-PAGE separation and transfer. Fig. 2*B* shows that the 167-kDa protein (*arrow*) could be digested by trypsin (*lanes 3* and *4*) only in the presence of detergent (*lane 4*) and that the detergent *per se* had no effect on the reactivity of the protein (*lanes 1* and *2*).

In the molecular mass region of 165–170 kDa, SR terminal cisternae are enriched in two proteins, the 165-kDa sarcalumenin (22, 23) and the 170-kDa histidine-rich protein (24, 25). Fig. 3*B* shows an SDS-PAGE separation of proteins from SR terminal cisternae, stained with Stains-all. Three polypeptides can be clearly identified in the 165–170-kDa range. Only the lower and higher molecular mass species underwent a metachromatic shift, while the intermediate-sized protein (*arrow*) maintained the original pink stain. Fig. 3*B* shows that the 167-kDa protein can be easily distinguished from histidine-rich protein and sarcalumenin on two-dimensional gels, owing to its slightly higher pI.

We have further purified the 167-kDa protein from SR terminal cisternae by extraction at pH 11 (see "Experimental Procedures" for details). The extract, containing histidine-rich protein, sarcalumenin, and the 167-kDa protein (data not shown), was neutralized and further fractionated through an IDA-Zn^{2+}-agarose column. As expected (26), sarcalumenin was recovered in the unbound fraction together with most of the 167-kDa protein, while histidine-rich protein remained bound to the column and could only be eluted with 0.1 M EDTA (data not shown). The flow-through fraction of the IDA-Zn^{2+}-agarose column was finally dialyzed against 100 mM NaCl, 10 mM Tris, pH 8.5, 2 mM dithiothreitol, and 0.1 mM PMSF; and under these conditions, a precipitate formed that contained the 167-kDa protein, but not sarcalumenin, as judged by SDS-PAGE (Fig. 3*D*) and two-dimensional electrophoresis (Fig. 3*C*). Note that

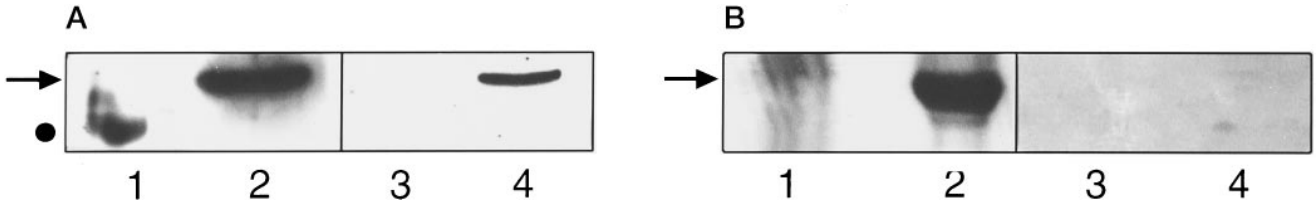

FIG. 4. **Reactivity of fibroblast integrins and the 167-kDa protein with $\alpha_5\beta_1$-integrin and 167-kDa peptide antisera.** *A*, extracts from normal rat kidney fibroblasts (*lanes 1* and *3*) and membrane fractions enriched in terminal cisternae from rabbit fast-twitch skeletal muscle SR (*lanes 2* and *4*) were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with (i) anti-$\alpha_5\beta_1$-integrin antibody at a 1:100 dilution (*lanes 1* and *2*) or (ii) antibodies purified from the same serum onto immobilized 167-kDa protein (*lanes 3* and *4*). *B*, extracts from normal rat kidney fibroblasts (*lanes 1* and *3*) and fractions enriched in the 167-kDa protein from rabbit slow-twitch skeletal muscle (*lanes 2* and *4*) were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with (i) a polyclonal antiserum against the 167-kDa peptide (*lanes 1* and *2*) or (ii) an irrelevant antiserum (*lanes 3* and *4*) at a 1:100 dilution. The positions of β_1-integrin (●) and the 167-kDa protein (→) are indicated. Note that the 167-kDa protein-specific antibodies do not cross-react with β_1-integrin, but only recognize the 167-kDa protein.

only one protein of 167 kDa could be identified, which did not undergo a color shift with the Stains-all dye (*arrows*). Western blotting of this preparation confirmed its identity to the 167-kDa protein (data not shown).

We next used two-dimensional gel separation followed by transfer to nylon membranes for sequencing the N terminus of the 167-kDa protein, which yielded the sequence *X*-*X*-Phe-Gly-Tyr-Thr-Gly-Leu, and a trypsin cleavage fragment, which yielded the sequence *X*-Leu-Gln-Val-Thr-Trp-Arg-Ile-Thr-Asn. Surprisingly, these sequences did not match any known integrins or any sequence in the Swiss and EMBL data bases. These findings indicate that 167-kDa protein is a novel, muscle-specific protein of the SR terminal cisternae that has probably escaped attention so far because of its comigration with sarcalumenin.

To understand the basis for its apparent cross-reactivity with the anti-$\alpha_5\beta_1$-integrin antiserum, we followed two strategies. In the first, we prepared rabbit antibodies against the synthetic peptide *X*-Leu-Gln-Val-Thr-Trp-Arg-Ile-Thr-Asn, modeled on the 167-kDa protein unique sequence. In the second, we used immobilized 167-kDa protein as an affinity matrix to purify specific antibodies from the anti-$\alpha_5\beta_1$-integrin antiserum. We then used the anti-peptide and affinity-purified antibodies to probe extracts from both rat 3T3 fibroblasts and a 167-kDa protein-containing muscle fraction in Western blots.

Fig. 4*A* shows that while the anti-$\alpha_5\beta_1$-integrin antiserum recognized both the β-integrin subunit of normal rat kidney fibroblasts (*lane 1*) and the 167-kDa protein in muscle (*lane 2*), the affinity-purified antibody failed to recognize β-integrin (*lane 3*), while, as expected, it still reacted with the 167-kDa protein (*lane 4*). Likewise, the anti-peptide antiserum recognized the 167-kDa protein in a membrane fraction from rabbit slow-twitch skeletal muscle (Fig. 4*B*, *lane 2*), but not β-integrin (*lane 1*). Note that a preimmune serum did not stain either β-integrin (*lane 3*) or the 167-kDa protein (*lane 4*). We must conclude that the 167-kDa protein and β-integrins are not antigenically related.

The basis for the reactivity of our anti-integrin antiserum with the 167-kDa protein remains obscure. One possibility is that a protein related to the 167-kDa protein or an ~140-kDa proteolytic product was present in the material used for immunization. Contamination by the "native" 167-kDa protein appears extremely unlikely because (i) the integrin was purified by sequential chromatographic steps on wheat germ agglutinin and DEAE-cellulose (neither of which binds the 167-kDa protein) prior to affinity chromatography on a fibronectin peptide and elution with GRGDSP (18), and (ii) only a single diffuse band of ~140 kDa could be detected by silver staining after SDS-PAGE separation of the material used for immunization under reducing conditions (data not shown, but see Ref. 18). However, integrin recognition motifs have been found in several intracellular muscle proteins, including the N-terminal REDV sequence of dystrophin (17), and in SR intraluminal proteins, as in the case of $\alpha_2\beta_1$-integrin-calreticulin interactions (27).

The function of the 167-kDa protein remains to be elucidated and can only be a matter of speculation at present. Its muscle-specific expression and subcellular distribution, however, suggest a role in muscle cell physiology, which could be related to regulation of Ca^{2+} homeostasis. Determination of the primary sequence of the 167-kDa protein is under way in our laboratories and should contribute to resolving the quest for its function.

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A Novel Muscle Protein Located inside the Terminal Cisternae of the Sarcoplasmic Reticulum

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