

Identification of Sarcolemma-associated Antigens with Differential Distributions on Fast and Slow Skeletal Muscle Fibers

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Abstract. We have identified three sarcolemma-associated antigens, including two antigens that are differentially distributed on skeletal muscle fibers of the fast, fast/slow, and slow types. Monoclonal antibodies were prepared using partially purified membranes of adult chicken skeletal muscles as immunogens and were used to characterize three antigens associated with the sarcolemma of muscle fibers. Immunofluorescence staining of cryosections of adult and embryonic chicken muscles showed that two of the three antigens differed in expression by fibers depending on developmental age and whether the fibers were of the fast, fast/slow, or slow type. Fiber type was assigned by determining the content of fast and slow myosin heavy chain. MSA-55 was expressed equally by fibers of all types. In contrast, MSA-slow and MSA-140 differed in their expression by muscle fibers depending on fiber type. MSA-slow was detected exclusively at the periphery of fast/slow and slow fibers, but was not detected on fast fibers. MSA-140 was detected on all fibers but fast/slow and slow fibers stained more intensely suggesting that these fiber types contain more MSA-140 than fast fibers. These sarcolemma-associated antigens were developmentally regulated in ovo and in vitro. MSA-55 and MSA-140 were detected on all

primary muscle fibers by day 8 in ovo of embryonic development, whereas MSA-slow was first detected on muscle fibers just before hatching. Those antigens expressed by fast fibers (MSA-55 and MSA-140) were expressed only after myoblasts differentiated into myotubes, but were not expressed by fibroblasts in cell culture. Each antigen was also detected in one or more nonskeletal muscle cell types: MSA-55 and MSA-slow in cardiac myocytes and smooth muscle of gizzard (but not vascular structures) and MSA-140 in cardiac myocytes and smooth muscle of vascular structures. MSA-55 was identified as an M_r 55,000, nonglycosylated, detergent-soluble protein, and MSA-140 was an M_r 140,000, cell surface protein. The M_r of MSA-slow could not be determined by immunoblotting or immunoprecipitation techniques. These findings indicate that muscle fibers of different physiological function differ in the components associated with the sarcolemma. While the function of these sarcolemma-associated antigens is unknown, their regulated appearance during development in ovo and as myoblasts differentiate in culture suggests that they may be important in the formation, maturation, and function of fast, fast/slow, and slow muscle fibers.

SKELETAL muscles of vertebrates are composed of a heterogeneous population of muscle fibers having distinct biochemical, morphological, and physiological properties. While a spectrum of muscle fiber types exists in skeletal muscles, three general types of fibers, fast, mixed fast/slow, and slow, can be identified in adult avian muscles based on their content of fast and slow isoforms of myosin heavy chains (15, 26, 49). Muscle fiber types can also be distinguished by histochemical ATPase staining (4, 5, 8, 9), membrane resistances (22, 38), calcium transport systems (11, 23, 54), mechanisms of energy metabolism (18, 29), patterns of innervation (30, 35), and by the presence of distinct isoforms of other myofibrillar proteins (16, 46, 51). Many ultrastructural features of the sarcolemma and intracellular membrane

systems differ among the three fiber types (19, 39, 40, 54, 61) but the biochemical basis for many of the structural differences have not been identified.

Specific identification of the different muscle fiber types in situ based upon cell membrane components has not been accomplished, although changes in some sarcolemma-associated antigens of muscle fibers throughout myogenesis have been demonstrated. For example, both polyclonal antisera and monoclonal antibodies have been used to identify sarcolemma-associated antigens of skeletal muscle cells (10, 21, 25, 31, 33, 42, 70). Some of these antigens are expressed only by muscle cells at specific developmental stages, while others are expressed at all stages of muscle development. A few of the antigens undergo dramatic quantitative changes in their

topographic distribution on cells as myoblasts differentiate into myotubes (33, 36, 37). Not all skeletal muscle sarcolemma antigens or epitopes are confined to skeletal muscle cells; some are detected on smooth or cardiac muscle cells and on nonmuscle cells (17, 43, 69). None of the sarcolemma antigens that have been identified using immunological probes distinguish among fast, fast/slow, and slow muscle fibers.

To determine if there are sarcolemma-associated components that distinguish fast, fast/slow, and slow skeletal muscle fibers from one another and to identify sarcolemma-associated components of cells of the fast, fast/slow, or slow myogenic lineages (48, 49) we isolated monoclonal antibodies that recognize antigens associated with the sarcolemma of chicken skeletal muscle fibers. Antibodies to components of the epimysium, perimysium, and myofibrillar proteins were specifically excluded. Those selected for further study bound at the periphery of one or more skeletal muscle fiber types in cross sections and the antigens detected by each antibody were designated as muscle sarcolemma-associated antigens (MSAs).¹ Attention was directed to antibodies reacting with three classes of antigens: those that were expressed equally by all fiber types; those that differed quantitatively in expression between fiber types, though were expressed by all fiber types; and those that were expressed only by specific fiber types. Three monoclonal antibodies, one reacting with an antigen from each class, were selected for characterization of the antigens of muscle fibers formed *in vivo* and in cell culture. MSA-55 was expressed equally by all skeletal muscle fibers; MSA-140 was expressed by all fibers, but it was expressed preferentially by slow and fast/slow fibers; and MSA-slow was expressed only by slow and fast/slow fibers of the adult chicken.

Materials and Methods

Monoclonal Antibodies

Monoclonal antibodies (mAb's) to chicken sarcolemma components were produced by hybridomas formed by fusion of spleen cells of BALB/c mice immunized with sarcolemma preparations of adult chicken skeletal muscles and P3-NSI/1 Ag 4-1 myeloma cells. Immunization, cell fusion, and subcloning were performed as described by Oi and Herzenberg (52). Hybridomas were screened for the production of antibodies that bound to the periphery of muscle fibers by immunohistochemical staining of transverse sections of the pectoralis major, anterior latissimus dorsi (ALD), and sartorius muscles of adult chickens. Biotinylated horse anti-mouse immunoglobulin and avidin-horseradish peroxidase were used as the secondary reagents (Vectastain ABC reagent, Vector Laboratories, Inc., Burlingame, CA). Monoclonal antibodies S58 and F59, which recognize the slow and fast classes of myosin heavy chain isoforms, respectively, have been previously characterized (15, 49). A mAb prepared in this laboratory that is specific for *Escherichia coli* β -galactosidase was used in control experiments.

Sarcolemma Isolation

Crude sarcolemma preparations from either the pectoralis major muscle or the combined muscles of the hind limbs of adult white Leghorn chickens that contained fast, fast/slow, and slow fibers were prepared according to the initial steps in the procedure of Seiler and Fleischer (63). Particulate material sedimenting in the sucrose density gradient at the interface between

1. *Abbreviations used in this paper:* ALD, anterior latissimus dorsi; BSA-HS, 2% bovine serum albumin in PBS containing 2% horse serum; DME-HS, Dulbecco's modified Eagle's medium containing 10% horse serum; MSA, muscle sarcolemma-associated antigen; PLD, posterior latissimus dorsi.

the 17% sucrose and 23% sucrose layers contained the highest specific activity of ouabain-sensitive Na⁺-K⁺ ATPase (58) and was chosen as the sarcolemma fraction used for immunization of BALB/c mice.

Immunofluorescence Analysis of Cryosections and Muscle Cell Cultures

Tissues were frozen in melting isopentane and 10- μ m sections were cut and transferred to gelatin-coated glass slides. The sections were air dried, rinsed briefly in PBS (10 mM sodium phosphate, pH 7.4, containing 150 mM sodium chloride), fixed by incubation either in 3.7% formaldehyde in PBS (5 min) (for sections to be reacted with the mAb to MSA-140) or in 70% ethanol (4 min) (for sections to be reacted with the mAb's to MSA-55 and MSA-slow), rinsed briefly in PBS, and blocked by incubation for 30-60 min in 2% BSA in PBS containing 2% horse serum (BSA-HS). (Sections stained with F59 and S58 were fixed for 4 min in 100% ethanol.) Sections were incubated with hybridoma supernatants overnight at 4°C and antibody binding was visualized by fluorescence after incubation for 2-4 h at room temperature with biotinylated anti-mouse immunoglobulin (Vector Laboratories, Inc., Burlingame, CA) diluted 1:200 in BSA-HS followed by incubation for 1 h in Texas Red-conjugated streptavidin (Bethesda Research Laboratories, Gaithersburg, MD) diluted 1:400 in BSA-HS. Slides were washed with PBS after incubation with each reagent. Coverslips were mounted in Aquamount (Lerner Laboratories, New Haven, CT) and immunofluorescence was detected using a Zeiss microscope equipped with epifluorescence optics. The micrographs were prepared using Kodak Tri-X film.

Immunofluorescence staining of myoblasts and myotubes in culture was performed at 4°C. Incubation of cells with the antibodies was performed using either unfixed cells or cells that had been fixed by a 5-min incubation in 3.7% formaldehyde in PBS followed by 3 min in 50% ethanol. Cells were washed briefly in DME containing 10% horse serum (DME-HS) and incubated for 1-2 h with hybridoma supernatants diluted in DME-HS. Dishes were washed three times with cold DME-HS and incubated 1 h in biotinylated horse anti-mouse IgG diluted 1:200 in DME-HS. Antibody binding was visualized by fluorescence after incubation of the cultures with Texas Red-conjugated streptavidin diluted 1:200 in DME-HS. Before use, the biotinylated immunoglobulin and the Texas Red-conjugated streptavidin solutions were preincubated with unfixed muscle cell cultures for 1-2 h at 4°C to reduce nonspecific binding of the secondary reagents. After incubation with Texas Red-conjugated streptavidin, the cultures were washed, postfixed in 3.7% formaldehyde in PBS (5 min) followed by 50% ethanol (3 min), and coverslips were mounted using Aquamount.

Muscle Cell Culture and Radiolabeling

Muscle cell cultures were prepared as described by O'Neill and Stockdale (53). Cells from day 12 *in ovo* embryonic pectoral muscle were isolated by trypsinization and plated at a density of 1.0-1.6 $\times 10^4$ cells/cm² on collagen-coated dishes in Eagle's minimal essential medium containing 10% horse serum, 2.5% chick embryo extract, 2 mM glutamine, and 1% antibiotics (penicillin, streptomycin, fungizone). For immunoprecipitation, proteins were biosynthetically labeled for 15 h with 10 μ Ci/ml [³⁵S]methionine (specific activity = 130 mCi/mmol, Amersham Corp., Arlington Heights, IL) or 100 μ Ci/ml [³H]4,5-leucine (specific activity = 133 Ci/mmol, Amersham Corp.) on day 6 of culture in medium prepared with methionine-free or leucine-free Eagle's minimal essential medium, respectively. Muscle cell surface proteins were labeled by lactoperoxidase-catalyzed radioiodination of unfixed myotubes (45). Cells were labeled *in situ* as monolayer cultures on 100-mm dishes using 0.5 mCi Na ¹²⁵I/dish (specific activity = 13.3 mCi/ μ g, Amersham Corp.). After iodination, the dishes were washed three times in cold saline and extracts of the muscle cultures were prepared using 1.0 ml of lysis buffer/100-mm dish as described below.

Preparation of Muscle Cell Extracts

Extracts of chicken muscles and of muscle cells grown *in vitro* were prepared using a lysis buffer containing 50 mM Tris Cl, pH 8.0, 150 mM NaCl, 5% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EGTA, 1 mM *N*-ethylmaleimide, 1 mM *o*-phenanthroline, 1 mg/ml leupeptin, and 0.1 μ g/ml pepstatin. Muscles were dissected, minced with a razor, and homogenized in a Dounce homogenizer (15 strokes) in ice cold lysis buffer (5 ml buffer/g of tissue). The tissue homogenates were maintained on ice for 30 min and clarified by centrifugation for 1 h at 100,000 g. The supernatant fraction was collected, filtered

Table I. Tissue Distribution, Developmental Expression, and Biochemical Characteristics of the Sarcolemma-associated Antigens

	Antigen		
	MSA-55	MSA-slow	MSA-140
Adult Tissue Distribution*			
Skeletal Muscle			
Fast fibers	+++	—	+‡
Fast/slow fibers	+++	+	++
Slow fibers	+++	++	++
Cardiac muscle			
(ventricle)	++	++	++
Smooth muscle (gizzard)	+	++	—
Vascular structures	—	—	+
Nerves	—	—	+
Liver	—	—	—
Developmental Expression§			
Myoblasts in vitro	No	No	No
Myotubes in vitro	Yes	No	Yes
Myotubes in ovo	Yes	No	Yes
Biochemical Characteristics			
	55-kD protein (not glycosylated)	?	140-kD protein

* Relative intensity of immunofluorescence is designated from high (+++) to not detectable immunofluorescence (—).

‡ Relative intensity of immunofluorescence varied depending on the method of fixation of the tissue sections.

§ The presence or absence of the antigens on muscle cells in vitro or on embryonic muscle fibers in vivo is indicated.

through a 0.22- μ m filter, and stored at -70°C . Cells on tissue culture dishes were rinsed twice in Hanks' balanced salt solution (HBSS) and scraped from the dishes into 5 ml HBSS using a Teflon policeman. Cells from ten 100-mm dishes were pooled and homogenized in a tight-fitting Dounce homogenizer (10 strokes) in 3 ml cold lysis buffer. The cell extract was clarified and filtered as described above.

Gel Electrophoresis and Immunoblotting

One-dimensional SDS PAGE was performed as described by Laemmli (41) in either 8 or 10% acrylamide gels. Electrophoretic transfer of proteins from the gels to nitrocellulose was performed as described by Towbin et al. (67). For detection of transferred proteins with the mAb's, the nitrocellulose was first incubated with 2% nonfat dried milk in PBS containing 2% horse serum followed by incubation for 4 h in the monoclonal antibodies diluted in the milk solution. The nitrocellulose was washed in PBS and incubated in ^{125}I -labeled anti-mouse immunoglobulin [F(ab)₂ fragment; specific activity = 500–2,000 Ci/mmol, Amersham Corp.], diluted to a concentration of 0.25 $\mu\text{Ci}/\text{ml}$ in the milk solution. Finally, the blots were washed and exposed to XAR film at -70°C for 3 d using an intensifying screen.

Immunoprecipitation

Antigens were isolated from the radiolabeled cell extracts by incubating the extracts with mAb's followed by incubation with goat anti-mouse immunoglobulins conjugated to Sepharose resin (Zymed Laboratories, San Francisco, CA). Monoclonal antibody (1 ml of hybridoma culture medium) was incubated for 4–6 h at 4°C with 1.0 ml of the radiolabeled muscle cell extract. Immunoglobulin-coupled Sepharose resin (100 μl of a 1:1 suspension in lysis buffer) was added and the incubation continued overnight. To decrease nonspecific binding of radiolabeled proteins to the Sepharose resin, the resin was treated with a nonradioactive extract of muscle cells for 2–4 h at 4°C before use. Likewise, the radioactive cell extract was treated for 1 h at 40°C with a 100- μl aliquot of the resin before use in the immunoprecipitation reaction to remove any components that nonspecifically bind to the resin. The antigen-immunobead complex was washed four times in lysis buffer containing 0.5 M sodium chloride, washed twice in lysis buffer containing 0.1% SDS (wt/vol), and washed once in lysis buffer alone. Antigens were removed from the resin by boiling 5 min in electrophoresis sample buffer and analyzed by electrophoresis in 8, 10, or 12% polyacrylamide gels. The gels were fixed and stained with Coomassie Blue, impregnated with Enlightening fluorography enhancer (New England Nuclear, Boston, MA), dried under vacuum, and exposed to XAR film for 1–4 wk.

Results

Antigens closely associated with the sarcolemma of muscle fibers can distinguish mixed fast/slow and slow fibers from fast fibers. Immunofluorescence analysis of cryostat sections of embryonic, post hatch, and adult tissues was used to study the expression of three antigens, MSA-55, MSA-140, and MSA-slow, that are associated with the sarcolemma of the adult chicken muscle fibers. One of the antigens (MSA-55) was expressed equally by all skeletal muscle fibers, while two of the antigens (MSA-slow and MSA-140) differed in their expression by muscle fibers depending on the type of fiber. MSA-slow was expressed exclusively by slow muscle fibers and MSA-140 was expressed to a greater extent by fibers containing slow myosin heavy chain (fast/slow and slow fibers) than by fibers containing only fast myosin heavy chain. Table I summarizes the results of immunohistochemical and biochemical analyses of MSA-55, MSA-slow, and MSA-140 to be described below. The antigens were designated MSA (for muscle sarcolemma-associated antigen) followed by a description (i.e., 55 for M_r 55,000 or slow for fiber type) characteristic of each antigen. The antigens were defined as sarcolemma-associated antigens based on their localization by light microscopy at the periphery of muscle fibers in cross section. Thus, these antigens may be components of the muscle fiber plasma membrane, the basal lamina, or the intracellular cytoskeleton subjacent to the plasma membrane. For the purpose of this report, we have defined the antigens as any component reacting with the mAb's we have developed; the epitope recognized by a given antibody may be present on several forms of a protein and thus, may not be restricted to a single "antigen." Muscle fibers have been identified as fast, mixed fast/slow, or slow on the basis of the myosin heavy chain composition of the fibers (15, 48, 49).

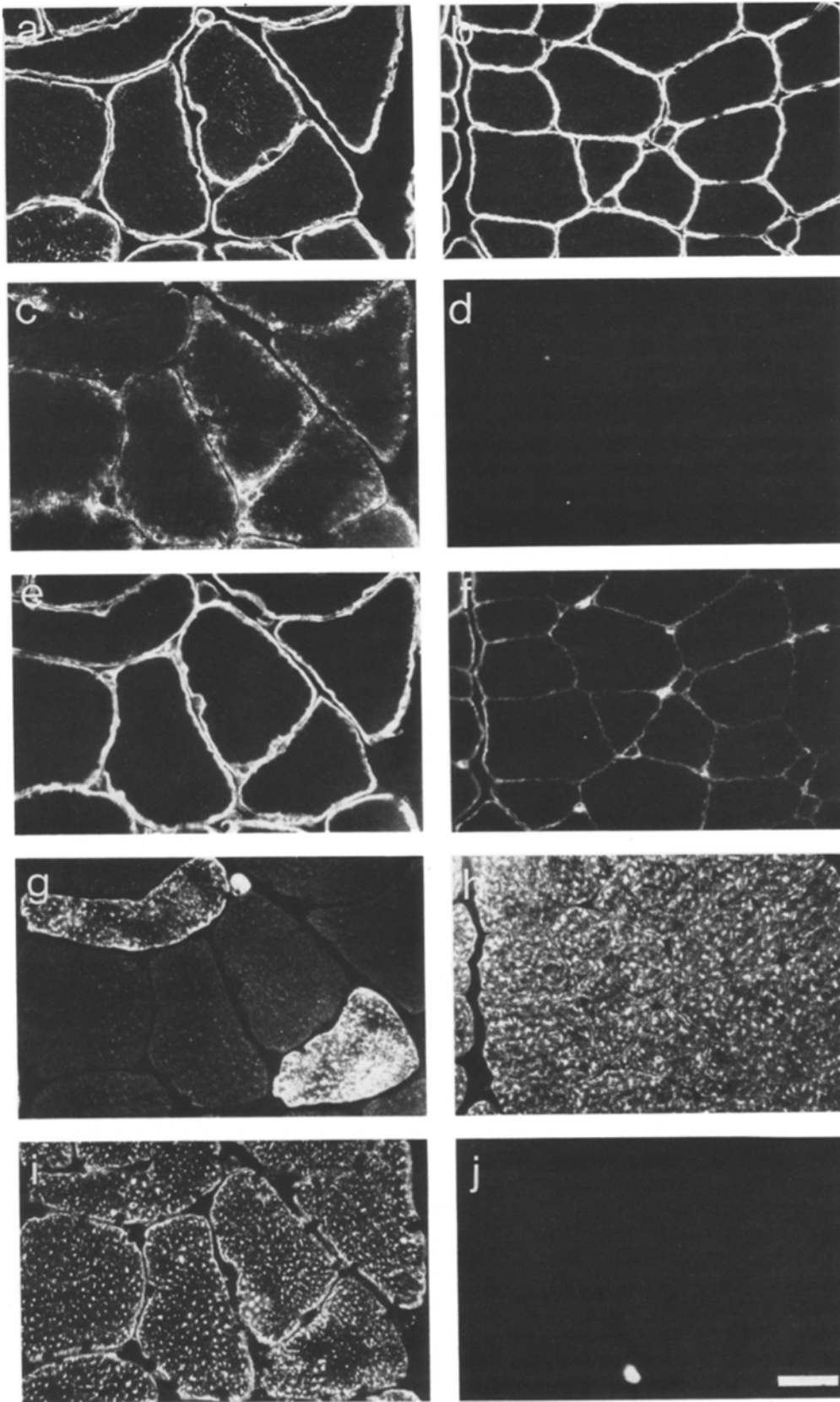


Figure 1. Immunohistochemical localization of MSA-55, MSA-slow, and MSA-140 and of fast and slow myosin heavy chain in adult chicken skeletal muscles. Serial transverse sections of the anterior latissimus dorsi (ALD) (*a, c, e, g, and i*) and the posterior latissimus dorsi (PLD) (*b, d, f, h, and j*) were reacted with mAb's to MSA-55 (*a and b*), MSA-slow (*c and d*), MSA-140 (*e and f*), fast myosin heavy chain (F59) (*g and h*), and slow myosin heavy chain (S58) (*i and j*). Antibody binding was detected by immunofluorescence using biotinylated anti-mouse immunoglobulins and Texas Red-streptavidin as described in Materials and Methods. MSA-55 was detected on all fibers of the ALD and PLD (*a and b*); MSA-slow was detected only on slow myosin-containing fibers of the ALD (*c and d*). MSA-140 was detected on all fibers but was most prominent on slow fibers of the ALD (*e and f*). Bar, 34 μ m.

Localization of the Antigens on Fast, Fast/Slow, and Slow Muscle Fibers In Vivo

Fig. 1 shows the immunohistochemical localization of MSA-55, MSA-slow, and MSA-140 and of fast and slow isoforms

of myosin heavy chain in serial transverse cryosections of the predominantly slow anterior latissimus dorsi (ALD) muscle (Fig. 1, *a, c, e, g, and i*) and of the predominantly fast posterior latissimus dorsi (PLD) muscle (Fig. 1, *b, d, f, h,*

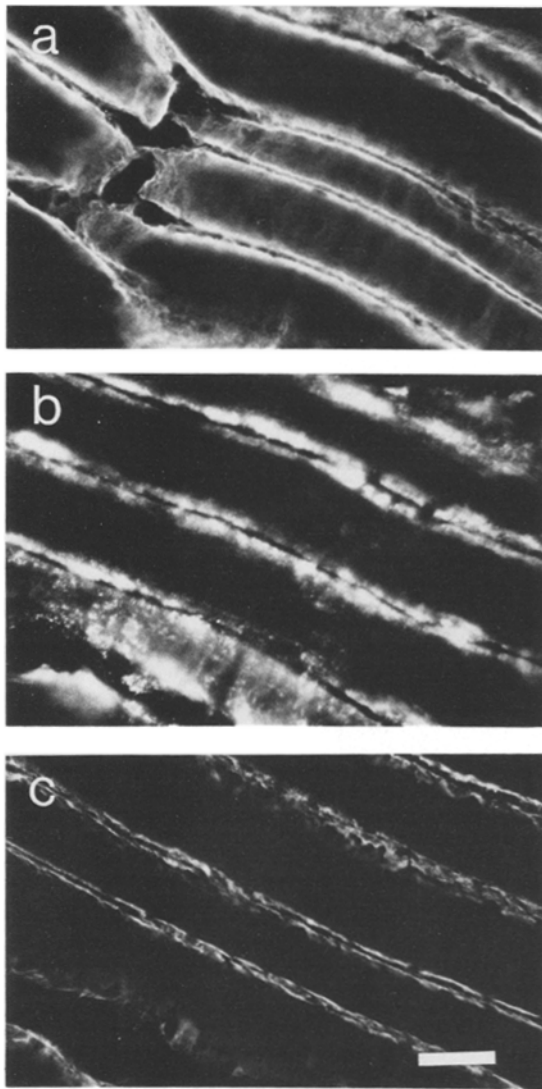


Figure 2. Immunohistochemical localization of MSA-55, MSA-slow, and MSA-140 in longitudinal sections of the ALD. MSA-55 (a) and MSA-140 (c) were distributed uniformly along the sarcolemma of each fiber. MSA-slow (b) was distributed in a diffuse, irregular pattern along the sarcolemma and appeared to extend into the sarcoplasm. Bar, 34 μ m.

and j). The distribution of each antigen along the length of fibers of the ALD is shown in longitudinally cut sections in Fig. 2. In cross sections of fibers, MSA-55 was uniformly distributed at the surface of all fibers of the ALD and PLD (Fig. 1, a and b). This antigen was also uniformly distributed along the length of the fiber surface as shown in the stained longitudinal sections (Fig. 2 a). The location of MSA-55 and the intensity of the immunofluorescence stain was the same in all skeletal muscles examined regardless of the muscle or fiber type. The antigen was detected exclusively on muscle fibers and was not detected in the epimysium or perimysium of muscle fascicles, on blood vessels, or in nerve bundles.

In contrast, MSA-slow was detected only around the periphery of slow muscle fibers (Fig. 1, c and d). All fibers of the predominately slow ALD reacted with the antibody to MSA-slow. MSA-slow was distributed in an irregular, stippled pattern at the periphery of the fibers; often the antigen

was concentrated in several intensely stained patches at the periphery of the fibers. In longitudinal sections of the ALD muscle (Fig. 2 b), the wide band of immunofluorescence detected along the surface of the fibers suggested that the antigen was located subjacent to the sarcolemma. Histochemical detection of acetylcholinesterase and succinate dehydrogenase or staining of serial sections with hematoxylin showed that MSA-slow was not located at the neuronmuscular junction, in mitochondria, or in nuclei of the muscle fibers (data not shown). Occasional fibers of the ALD were more lightly stained with the antibody to MSA-slow; these fibers were mixed fast/slow fibers that comprise a minor proportion of the muscle fibers of the ALD (48). Fibers of the medial adductor, a muscle of the thigh that contains predominantly slow myosin-containing fibers, reacted with the antibody to MSA-slow similarly to the fibers of the ALD. In contrast, antigen MSA-slow was not detected on any fibers of fast muscles such as the PLD (Fig. 1 d) or the pectoralis major. In addition, the antigen was not detected in the epimysium, perimysium, or on blood vessels, nerves, or any other structure in skeletal muscle.

MSA-140 also had a distinctive distribution on muscle fibers depending upon fiber type (Fig. 1, e and f). MSA-140 was distributed in a uniform, fine-lined pattern at the periphery of all muscle fibers, but the slow fibers of the ALD stained much more intensely than the fast fibers of the PLD (cf. Fig. 1, e and f). Slow fibers of the medial adductor also reacted intensely with the antibody while fast fibers of the pectoralis major were lightly stained. The antigen was distributed uniformly along the sarcolemma in longitudinal sections of the ALD (Fig. 2 c). In addition to its presence at the sarcolemma, MSA-140 was also detected in the tunica media of arteries and arterioles, in capillaries, in the endoneurium of myelinated axons, and in the perineural sheath of nerve bundles.

The distinct distribution of MSA-slow and MSA-140 on fast, fast/slow, and slow fibers was confirmed by immunohistochemical localization of these antigens in the sartorius, a muscle of the thigh composed of a mixture of fast, fast/slow, and slow fibers within the same fascicle. Fig. 3 shows the immunohistochemical location of MSA-55, MSA-slow, and MSA-140 and of fast and slow isoforms of myosin heavy chain in serial transverse sections of the adult sartorius muscle. As was observed in the ALD and PLD, MSA-55 was distributed uniformly around the periphery of all muscle fibers regardless of the myosin composition of the fibers (Fig. 3 c). Antibody to MSA-slow reacted only with the slow myosin-containing fibers of the sartorius (Fig. 3 d). The antigen was distributed on the fast/slow and slow fibers of the sartorius in a stippled pattern around the periphery of the fibers. Although the specificity of the antibody for slow myosin-containing fibers was retained, the intensity of the immunofluorescence staining was less than on slow and fast/slow fibers of the ALD. As in the ALD, the immunofluorescence staining of fast/slow fibers was usually less intense than slow fibers. Fast fibers of the sartorius did not react with the antibody to MSA-slow. MSA-slow had the same differential distribution on fast/slow and slow fibers of other muscles composed of a mixture of fiber types including the lateral adductor of the thigh and the "red" region of the pectoralis major. MSA-140 was distributed at the periphery of all fibers but the intensity of reaction of the antibody to MSA-140 with

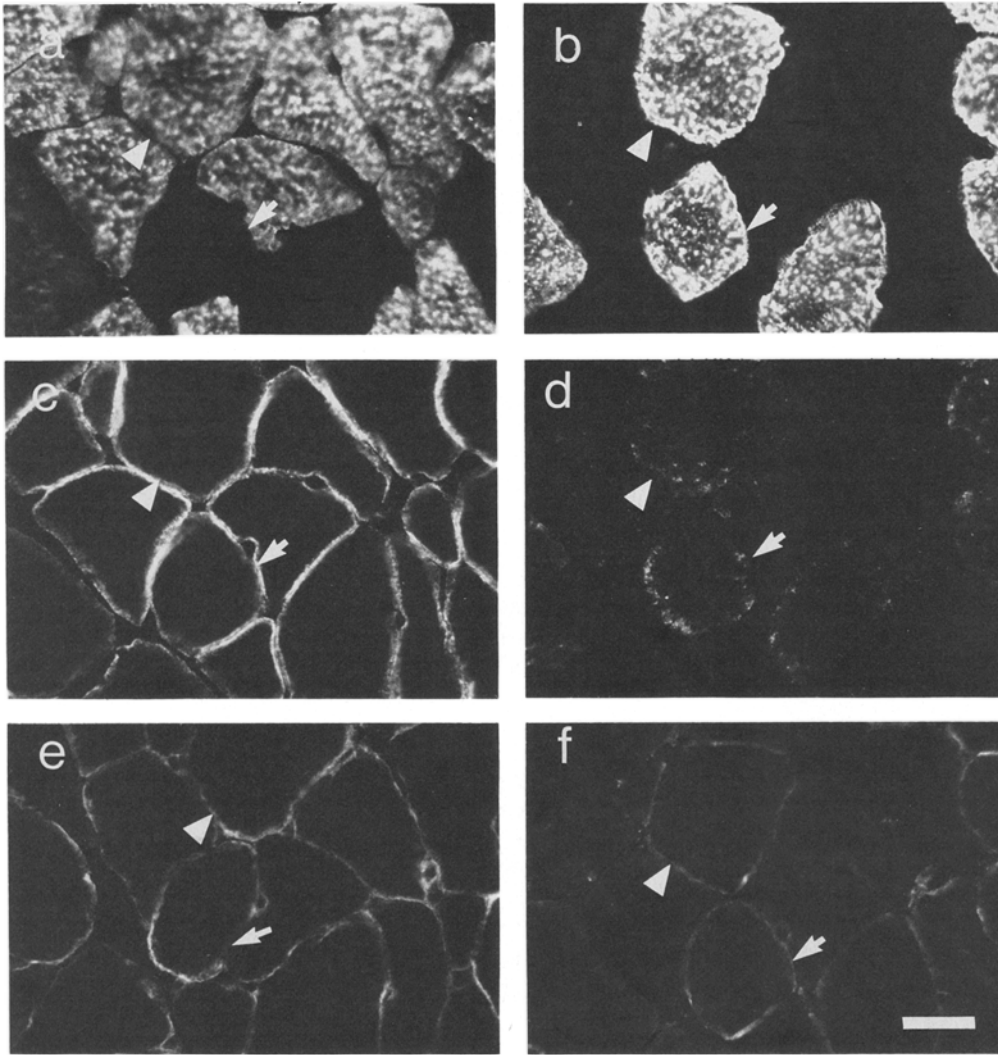


Figure 3. Immunohistochemical localization of MSA-55, MSA-slow, and MSA-140 on fast, slow, and mixed fast/slow fibers of the sartorius muscle. Serial transverse sections of the sartorius muscle were reacted with mAb's to fast myosin heavy chain (F59) (a), slow myosin heavy chain (S58) (b), MSA-55 (c), MSA-slow (d), and MSA-140 (e and f). Arrows identify a slow fiber that contains only slow myosin heavy chain and arrowheads identify a mixed fast/slow fiber that contains both fast and slow myosin. MSA-55 was detected at the periphery of all fibers (c). MSA-slow was detected only on slow and fast/slow fibers (d). MSA-140 was detected on all fibers of formaldehyde-fixed sections (e) but was detected only on slow and fast/slow fibers of 70% ethanol-fixed sections (f). Bar, 23 μ m.

fast, fast/slow, and slow fibers differed depending on the method of fixation of the tissue sections. When tissue sections from the sartorius, for example, were fixed in 3.7% formaldehyde (Fig. 3 e), all fibers appeared to bind the mAb to MSA-140 equally, however, when sections were fixed in 70% ethanol (Fig. 3 f), the intensity of staining was greater on fast/slow and slow fibers than on fast fibers. Thus MSA-140 distinguished fibers of the fast/slow and slow types from pure fast fibers.

Expression of MSA-55, MSA-140, and MSA-Slow by Developing Muscle Fibers

MSA-55, MSA-140, and MSA-slow were expressed by developing muscle fibers, and the regulation of expression of MSA-140 and MSA-slow changed during development in vivo. Cryosections of chick hindlimbs from day 8 in ovo through hatching (1 d post-hatch) were reacted with the mAb's to the three antigens (Fig. 4). MSA-55 and MSA-140 were detected on newly formed muscle fibers as early as day 8 in ovo and both antigens continued to be expressed on all muscle fibers through adulthood. In embryos aged 8 d in ovo and 12 d in ovo, MSA-55 had a slightly punctate distribution around the periphery of the developing fibers; as development proceeded, MSA-55 became uniformly distributed

around the fiber surface (Fig. 4 a, d, g, and j). MSA-55 was not detected on any structures other than muscle fibers of the embryonic hindlimb. MSA-140 was detected on muscle fibers of the limb early in development (Fig. 4, c, f, i, and l). Fibers of future fast and slow muscles of 8-, 12-, and 16-d embryos appeared to react equally with the antibody to MSA-140 but by 1 wk after hatching, the fast/slow and slow fibers of the medial adductor muscle were stained more intensely than fibers of the adjacent lateral adductor, a predominantly fast muscle. As in the adult, the antigen was also detected in arteries and nerves of developing limbs. In contrast, MSA-slow was not detected on any fibers of developing hindlimb muscles including the predominately slow medial adductor muscle until late in development (Fig. 4, b, e, h, and k). By the time of hatching, MSA-slow was detected on most fibers of the medial adductor as distinct patches at the periphery of the fibers (Fig. 4 k). No structures in the limb other than slow myosin-containing fibers reacted with the mAb to MSA-slow.

Expression of MSA-55, MSA-Slow, and MSA-140 by Muscle Cells in Cell Culture

To demonstrate that the antigens under study are produced by muscle cells themselves and to determine when in myogenic differentiation the antigens are first detected, myoblasts

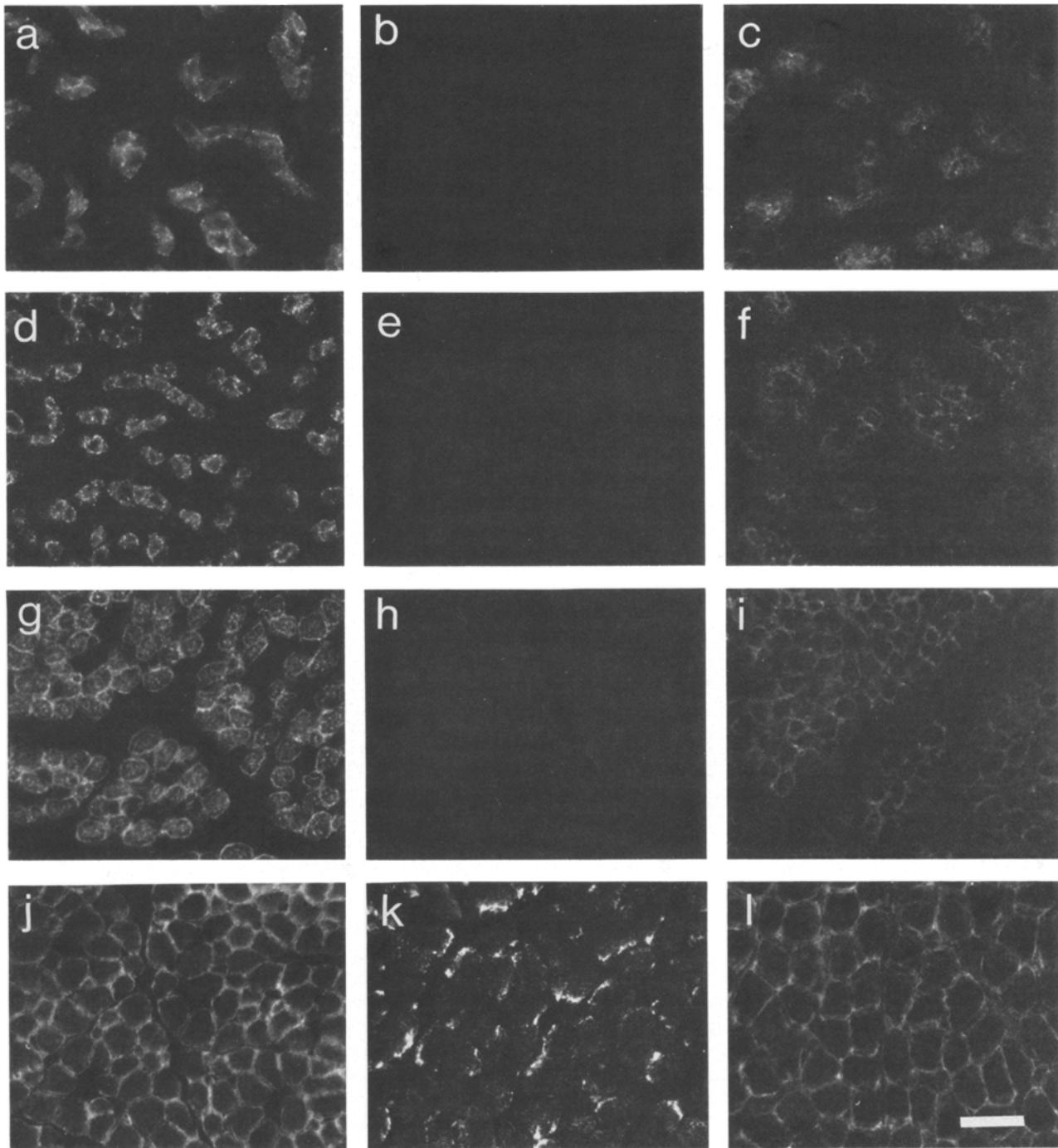


Figure 4. Immunohistochemical localization of MSA-55, MSA-slow, and MSA-140 in hindlimb muscles of developing chick embryos. Transverse sections of the hindlimbs of chick embryos aged 8 d (*a-c*), 12 d (*d-f*), 16 d (*g-i*) in ovo and 1 d post-hatch (*j-l*) were reacted with mAb's to MSA-55 (*a, d, g, and j*), MSA-slow (*b, e, h, and k*), and MSA-140 (*c, f, i, and l*). MSA-55 was detected at the periphery of all fibers from 8 d in ovo through hatching. MSA-slow was detected only after hatching in a punctate pattern at the periphery of slow and fast/slow fibers of the medial adductor (*k*). MSA-140 was detected at the periphery of all muscle fibers from 8 d in ovo through hatching. Bar, 23 μ m.

and myotubes in muscle cell cultures and muscle cell colonies were analyzed for the presence of each antigen using immunofluorescence analysis. Muscle cell cultures derived from cells of the pectoral muscle of chick embryos (12 d in ovo) were reacted with each antibody (Fig. 5). Cells were either fixed (Fig. 5, *a-f*) or unfixed (Fig. 5, *g-l*) before reaction with the mAb's. Previous studies of myotubes formed in

cultures derived from myoblasts of chicken embryos aged 12 d in ovo revealed that myotubes synthesize only fast myosin heavy chain(s) (2, 3, 49, 60). For this reason one might expect MSA-55 and MSA-140, but not antigen MSA-slow, to be expressed on myotubes formed in these cultures. Fig. 5 shows that, as expected, MSA-55 and MSA-140 are expressed by myotubes in culture, while MSA-slow is not.

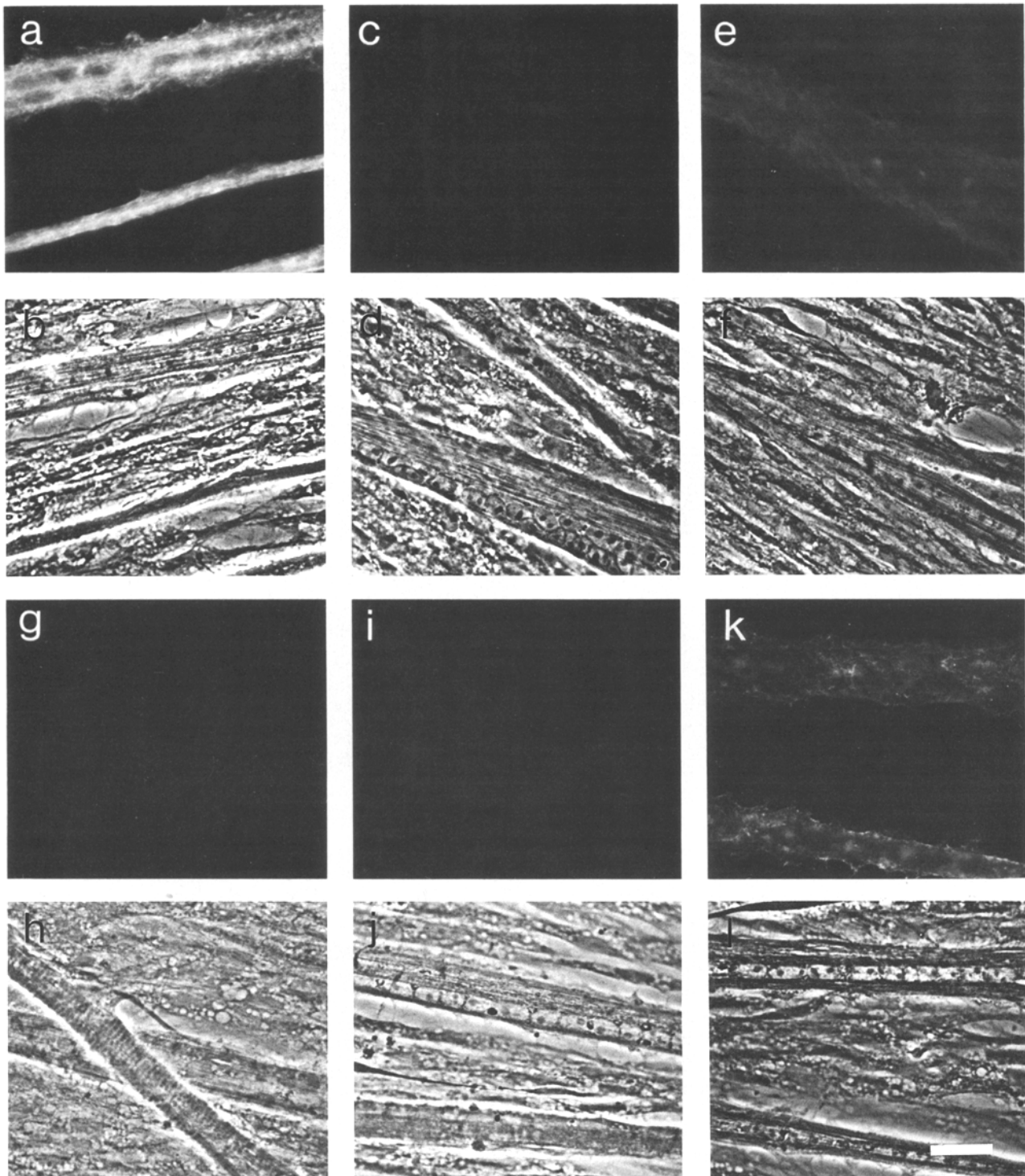


Figure 5. Immunocytochemical analysis of MSA-55, MSA-slow, and MSA-140 on muscle cells in culture. Cells from embryonic pectoral muscle were maintained in culture for 6 d and reacted with the mAb's either with or without fixation. Cells in *a-f* were fixed with 3.7% formaldehyde and ethanol before reaction with the mAb's. Cells in *g-l* were unfixed during all antibody incubations. Antibodies used were as follows: MSA-55 (*a, b, g, and h*); MSA-slow (*c, d, i, and j*); and MSA-140 (*e, f, k, and l*). Fluorescence micrographs are shown with their corresponding phase-contrast views below. MSA-55 was detected only on fixed myotubes and MSA-140 was detected in fixed and unfixed myotubes. MSA-slow was not expressed by myogenic cells in culture. Bar, 23 μ m.

MSA-55 and MSA-140 were detected on myogenic cells only after they differentiated. Neither unfused myoblasts nor fibroblasts reacted with the antibodies to MSA-55 or MSA-140, whereas myotubes stained intensely. Conditions for the

detection of these two antigens on myotubes differed. MSA-55 was detected on fixed, but not on unfixed myotubes (Fig. 5, *a-b, g-h*), suggesting that the antigenic site recognized by the antibody may not be accessible on the extracellular sur-

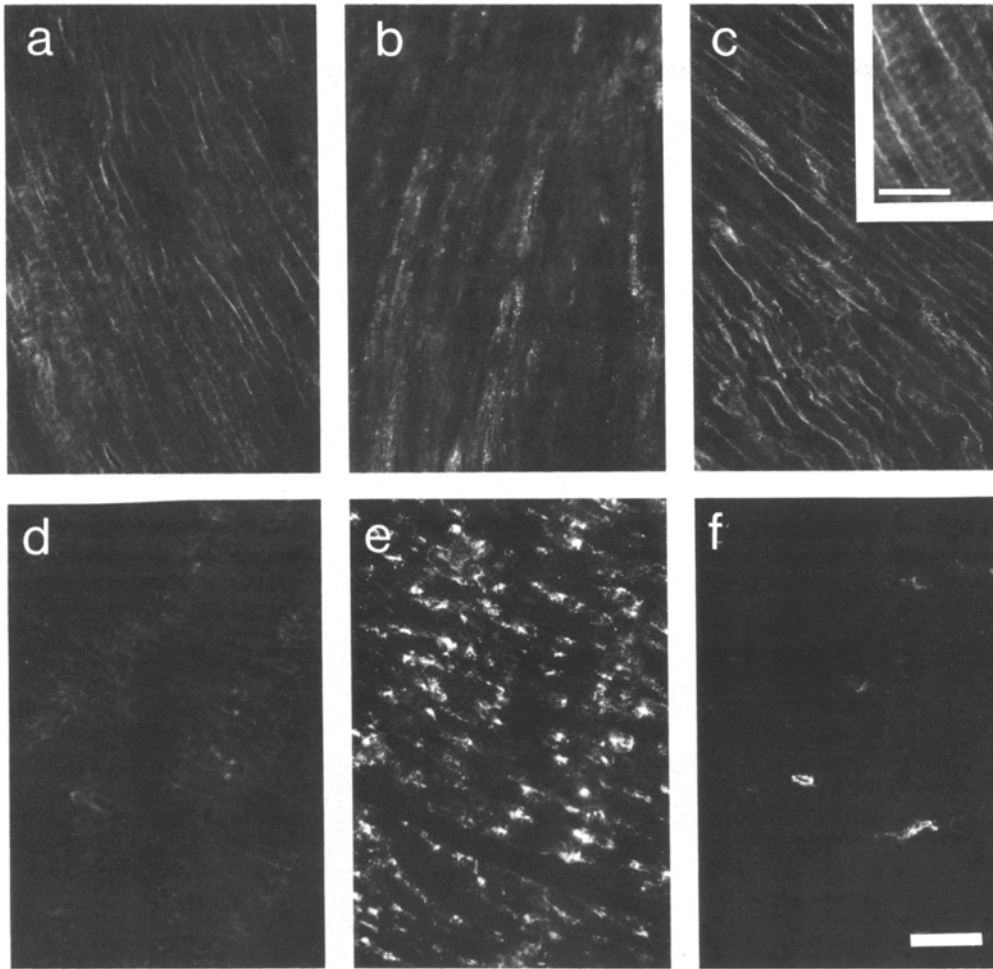


Figure 6. Immunohistochemical localization of MSA-55, MSA-slow, and MSA-140 in adult chicken ventricle and gizzard. Cryosections of ventricle (a-c) and gizzard (d-f) from an adult chicken were reacted with mAb's to MSA-55 (a and d), MSA-slow (b and e), and MSA-140 (c and f). Cardiac ventricular muscle contained all three antigens; inset in c is a higher magnification showing the periodic distribution of MSA-140 in cardiac myocytes (bar, 10 μ m). Smooth muscle of gizzard contained MSA-55 and MSA-slow. MSA-140 was detected in the media of blood vessels of cardiac muscle and gizzard but not on the smooth muscle cells of the gizzard (f). Bar, 34 μ m.

face of the cell. The antigen was distributed in a fine network on most myotubes. MSA-140 was detected on both fixed and unfixed myotubes (Fig. 5, e-f, k-l), suggesting that the antigenic site recognized by the antibody is accessible on the extracellular surface of myotubes. MSA-140 was distributed in a stippled pattern along the length of all myotubes. MSA-slow was not detected on any cells in fixed or unfixed muscle cell cultures (Fig. 5, c-d, i-j). Analysis of muscle cell cultures that contained primarily unfused cells (i.e., after 1 and 2 d of culture) by immunofluorescence and analysis of suspensions of myoblasts and fibroblasts with a fluorescence-activated cell sorter confirmed that none of the antigens were expressed by mononucleated cells (data not shown).

Results of immunocytochemical analyses of myotubes and myoblasts in colonies formed by cloned myogenic cells prepared from embryonic pectoral muscle (12 d in ovo) or from limb buds (4 d in ovo) were identical to those of the mass cultures described above. Both MSA-55 and MSA-140 were detected only on myotubes and were not detected on myoblasts in the colonies or on cells of nonmyogenic colonies. Thus, these antigens are synthesized by the muscle cells themselves and their expression is dependent on differentiation. MSA-slow was not detected on myoblasts, myotubes, or other cells of the clonal cultures regardless of the source of cells used to prepare the cultures (see Discussion).

Distribution of MSA-55, MSA-Slow, and MSA-140 in Cardiac Muscle, Smooth Muscle, Vascular Structures, and Nerves

The distribution of MSA-55, MSA-slow, and MSA-140 were investigated in other tissues and cell types of the adult chicken including cardiac muscle, smooth muscle, peripheral nerves, arteries, and capillaries using immunofluorescence (Fig. 6). All three antigens were detected on cardiac muscle fibers of the adult heart ventricle. Both MSA-55 and MSA-140 were detected on cardiac muscle in a linear, track-like staining pattern (Fig. 6, a and c), suggesting that the antigens were located along the cellular membrane. (Because the orientation of the cardiac muscle cells is not completely longitudinal, the track-like staining is not always continuous.) While MSA-55 was distributed continuously along the periphery of the cardiac myocytes, the distribution of MSA-140 in cardiac muscle showed a periodic distribution with the same periodicity as the sarcomeres of the myofilaments ($\sim 2 \mu$ m) (Fig. 6 c, inset), suggesting that its distribution on cardiac myocytes was related to the organization of proteins of the myofilament. MSA-slow was detected in cardiac myocytes in a diffuse punctate pattern (Fig. 6 b) and the antigen was not confined to the region near the sarcolemma. The punctate distribution of MSA-slow was most striking in giz-

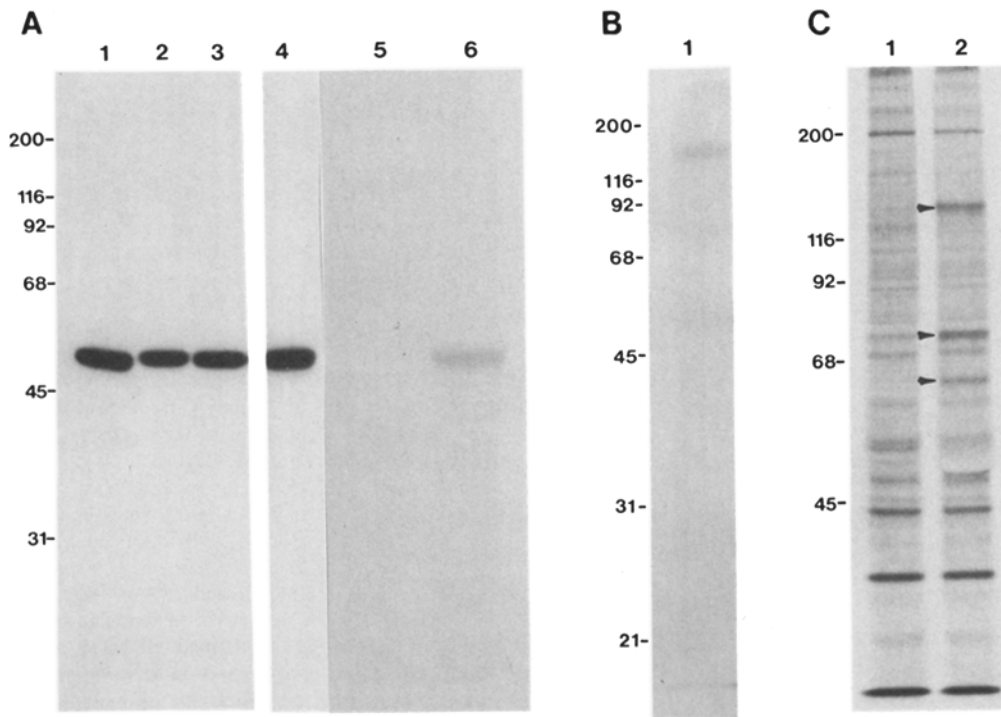


Figure 7. Biochemical characterization of MSA-55 and MSA-140 by immunoblotting and immunoprecipitation. (A) Immunoblotting analysis (lanes 1-4) and immunoprecipitation analysis (lanes 5 and 6) of MSA-55 in extracts of muscles and of muscle cell cultures. Aliquots containing 50 μ g of protein of extracts of the ALD (lane 1), PLD (lane 2), sartorius (lane 3), and an aliquot containing 100 μ g of protein of an extract of muscle cell cultures (lane 4) were subjected to SDS PAGE in 10% gels. Proteins were transferred to nitrocellulose and the blots were reacted with antibody to MSA-55 followed by reaction with 125 I-labeled anti-mouse IgG (Fab)₂ fragment. The antigen was identified as an M_r 55,000 protein in all extracts. Immunoprecipitation analysis (lanes 5 and 6) of MSA-55 was performed using extracts

of [35 S]methionine-labeled muscle cell cultures. Lane 5 shows control immunoprecipitation reaction using an mAb to β -galactosidase, and lane 6 shows detection of an M_r 55,000 protein using the mAb to MSA-55. The autoradiogram was exposed for 3 d. The positions of the molecular mass standards ($\times 10^{-3}$) are indicated on the left of the lanes: 200, myosin; 116, galactosidase; 92, phosphorylase B; 68, bovine serum albumin; 45, ovalbumin; 31, carbonic anhydrase; 21, soybean trypsin inhibitor. (B) Immunoprecipitation analysis of MSA-140 from extracts of myotubes radioiodinated in situ via lactoperoxidase-catalyzed iodination (lane 1). A single component of M_r 140,000 is detected in extracts of myotubes. SDS PAGE was performed in a 12% gel. The positions of the molecular mass standards are indicated on the left of the lane and correspond to the proteins described above. (C) Immunoprecipitation analysis of MSA-140 from extracts of [3 H]leucine-labeled myotube cultures (lanes 1 and 2). Lane 1 shows a control reaction using an mAb to β -galactosidase and indicates that some [3 H]leucine-labeled proteins bind nonspecifically to the immunobeads. Arrowheads in lane 2 identify three proteins having M_r 140,000, M_r 77,000, and M_r 67,000, that are specifically precipitated using the mAb to MSA-140. (The slight distortion in the mobility of proteins having an M_r \sim 50,000 is due to the presence of the immunoglobulin heavy chain of the mAb used in each immunoprecipitation reaction.) SDS PAGE was performed in 8% gels and the autoradiogram was exposed 1.5 mo. The positions of the molecular mass standards are indicated on the left of the lane as described above.

zard (Fig. 6 e). The distribution of this antigen as discrete patches within the smooth muscle of the gizzard resembled that of adherens junction or dense bodies, but MSA-slow does not have the same distribution as components of adherens junction or dense bodies such as vinculin, talin, and a 135-kD protein in chicken ventricle and gizzard (28). MSA-55 was barely detected on the smooth muscle of the gizzard (Fig. 6 d) and MSA-140 was detected only in the media of arteries and arterioles of the gizzard (Fig. 6 f). MSA-140 was also detected in the perineural sheath surrounding nerve bundles and was detected in the endoneurium around nerve axons in cross section (data not shown). None of the antigens were detected on ducts or parenchymal cells of liver from adult chickens (data not shown).

Biochemical Characterization of MSA-55 and MSA-140

MSA-55 and MSA-140 were characterized on nitrocellulose immunoblots and by immunoprecipitation. Immunoblot analysis was performed with detergent extracts of muscle cells grown in tissue culture and with detergent extracts of adult skeletal muscles. Immunoprecipitation analysis was performed on detergent extracts of cultured muscle cells that

had been radiolabeled in situ with 125 I by the lactoperoxidase reaction or biosynthetically labeled with [35 S]methionine or [3 H]leucine.

MSA-55 was identified as an M_r 55,000 component on immunoblots after SDS PAGE of extracts of adult skeletal muscles and of extracts of muscle cultures (Fig. 7, A, lanes 1-4). The equal intensity of the bands of the autoradiogram corresponding to MSA-55 in aliquots of extracts containing 50 μ g of protein of the ALD, PLD, and sartorius muscles suggested that comparable amounts of MSA-55 were expressed by all muscle fiber types. The 55-kD protein was also isolated by immunoprecipitation of extracts of muscle cultures biosynthetically labeled with [35 S]methionine (Fig. 7 A, lane 6) but was not detected in immunoprecipitates when muscle cultures were radiolabeled with 125 I (not shown). This result and the immunocytochemical staining results in Fig. 5 suggested that the epitope detected by the mAb to MSA-55 was not exposed on the extracellular surface of the sarcolemma. MSA-55 was not likely to be a glycoprotein because it did not bind to concanavalin A-Sepharose and its mobility in SDS gels was not altered by treatment with endoglycosidase F.

MSA-140 was identified as an M_r 140,000 protein by SDS

PAGE of immunoprecipitated proteins of myotube cultures in which the unfixed cells were radiolabeled in situ with ^{125}I by the lactoperoxidase reaction (Fig. 7 B, lane 1). This result and the observation that unfixed myotubes bound the antibody to MSA-140 suggested that the epitope detected by the mAb to MSA-140 was accessible on the extracellular surface of the myotubes. When myotube cultures were biosynthetically labeled with [^3H]leucine, the 140-kD protein was specifically immunoprecipitated from extracts of the muscle cultures (Fig. 7 C, lane 2). Two additional proteins of M_r 77,000 and M_r 67,000 were also identified. It is unlikely that the two low molecular weight proteins were degradation products of the larger 140-kD protein because several protease inhibitors were included in all buffers used in the immunoprecipitation reactions and they were not detected in extracts of ^{125}I -labeled cells. The 77- and 67-kD proteins may be integral membrane components that are noncovalently associated with MSA-140. No proteins were specifically immunoprecipitated by the antibody to MSA-140 when [^{35}S]methionine was used to biosynthetically label proteins of the muscle cells. MSA-140 was not detected on nitrocellulose immunoblots of SDS polyacrylamide gels containing proteins of adult muscle extracts or of muscle culture extracts.

Repeated attempts to identify MSA-slow on nitrocellulose immunoblots containing extracts of the ALD, of medial adductor, or of muscle cell cultures were unsuccessful. The epitope recognized by the antibody to MSA-slow may be sensitive to denaturation in the presence of SDS or may undergo conformational changes during solubilization that prevent it from reacting with the antibody. Reaction with the antibody could not be restored by omitting β -mercaptoethanol from the electrophoresis samples or by substituting a zwitterionic detergent (Empigen BB) for SDS during electrophoresis (44). As expected from the immunocytochemical analysis of muscle cells in culture, MSA-slow was not detected by immunoprecipitation of biosynthetically labeled muscle culture extracts.

Discussion

We have identified three developmentally regulated antigens associated with the sarcolemma of skeletal muscle fibers representing three different patterns of expression in fast, fast/slow, and slow fibers. MSA-55 was expressed by all skeletal muscle fibers; MSA-slow was expressed exclusively by fibers of newly hatched or older chickens that contained slow myosin; and MSA-140 was expressed by all fibers of the chick embryo, but to a greater extent by fast/slow and slow fibers than by fast fibers of adult chickens. The localization of these antigens by light microscopy was not precise, but mAb's to all three antigens bound at the periphery of the muscle fibers suggesting that the antigens they recognize were distributed near the sarcolemma. Whether these antigens are integral components of the plasma membrane, components of the extracellular basal lamina or components of the cytoskeleton subjacent to the plasma membrane, none of them is associated with the epimysium or perimysium of muscle fascicles and at least two of them are produced by the muscle cells themselves. They serve to identify fast, fast/slow, and slow fibers of adult muscles and differentiated cells of the myogenic lineage in early developing limbs and in cell cul-

ture. A summary of the biochemical properties and localization of each antigen in chicken tissues is given in Table I.

MSA-55 was detected only in muscle tissues and, in skeletal muscle, the antigen was expressed equally by all fiber types. MSA-55 was detected in the first muscle fibers (primary myotubes) to form in the developing embryo and on multinucleated myotubes as they formed in vitro. The antigen was distributed in a punctate pattern around the periphery of fibers formed early in development (8–12 d in ovo) but became uniformly distributed around the fiber periphery as the fibers matured. The developing bone, skin, and supporting matrix tissues of the limb did not contain MSA-55. It is likely that this nonglycosylated 55-kD protein is located on the cytoplasmic surface of the sarcolemma because myotubes in cell culture had to be fixed to detect the antigen and the antigen was not labeled with ^{125}I by iodination of myotubes in situ. The distribution of MSA-55 in muscle fibers is similar to that of muscle spectrin (47, 50) but, in contrast with MSA-55, greater amounts of spectrin have been detected in the ALD than the PLD (45). Vinculin, a 130-kD protein in the dense plaques of skeletal muscle fibers (55), cardiac myocytes (56), and smooth muscle cells (28), has a similar location to MSA-55 in cross sections of skeletal muscle fibers, but vinculin and MSA-55 do not have the same distributions in cardiac muscle and smooth muscle of the gizzard (28, 56). A recent study has shown there is less vinculin subjacent to the sarcolemma of the PLD than the ALD, whereas we detected approximately equal amounts of MSA-55 in the ALD and PLD by immunofluorescence staining and by immunoblotting analysis (64). In addition, MSA-55 is not likely to be desmin (55 kD) or vimentin (58 kD), since neither of these two intermediate filament proteins has the same location as MSA-55 in skeletal muscle fibers (32). While MSA-55 is not likely to be a known component of muscle fibers, it could be a component of the spectrin-based membrane-associated cytoskeleton, participate in binding myofilaments to the cellular membrane, or participate in linking the extracellular matrix and the intracellular cytoskeleton. Two recent reports have identified an actin-binding protein with a subunit molecular mass of 55 kD that is distributed near the plasma membrane in nonmuscle cells (68, 71). MSA-55 could serve the analogous function in striated muscle cells.

In skeletal muscle, MSA-slow was found exclusively on slow myosin-containing muscle fibers of both slow muscles (e.g., the ALD) and of mixed muscles (e.g., the sartorius). This antigen is the first example of a sarcolemma-associated component that is specific for slow or mixed fast/slow muscle fibers, and MSA-slow could be important in mediating the differential actions of fast, mixed fast/slow, and slow muscle fibers. The exact cellular location of the antigen was not determined, but its distribution in a broad band along the periphery of all slow fibers (Fig. 2) suggests that it is located on the intracellular side of the sarcolemma. The punctate distribution of MSA-slow in cardiac muscle and smooth muscle of the gizzard resembled the distribution of adherens junctions found in these cell types. However, MSA-slow does not have the same distribution as vinculin, talin, or a 135-kD protein known to be components of the adherens junctions in these tissues (27, 28). The distribution of MSA-slow in smooth muscle was also different from the distribution of filamin (65). MSA-slow was not detected in slow and fast/slow skeletal muscle fibers developing in vivo until near the

time of hatching, even though fibers containing slow myosin appear in the limb by 5 d of development in ovo (15). This antigen also was not detected in cultures of early muscle fibers that express slow myosin heavy chain (48, 49). These observations suggest that MSA-slow may be a sarcolemma-associated protein expressed during muscle fiber maturation rather than during muscle fiber formation and that factors extrinsic to the fiber may regulate its expression in vivo.

MSA-140 was accessible on the extracellular surface of myotubes formed in cell culture and is likely to be a protein of the sarcolemma or a protein of the basal lamina. While the amount of MSA-140 in extracts of fast and slow muscles could not be quantitated, there were dramatic differences in the intensity of the immunofluorescence stain on fibers in predominately fast or slow muscles. Slow fibers of the ALD stained more intensely than fast fibers of the PLD. Moreover, we have observed that MSA-140 on fast fibers of the sartorius was more labile to ethanol treatment than on fast/slow and slow fibers, suggesting that the organization of MSA-140 (and possibly other antigens) on the cell surface of muscle fibers may differ depending on the muscle fiber type. The molecular mass of ~140 kD is identical to that of the fibronectin receptor (57). It is unlikely that MSA-140 is the fibronectin receptor of skeletal muscle because it was present on myotubes (which do not bind fibronectin [12]) and was absent from myoblasts and fibroblasts (which do bind fibronectin [12]). Expression of MSA-140 was regulated during development since it was detected on all the earliest fibers to form in the developing limb, but fast and slow muscles were not distinguished by the intensity of the staining reaction until the time of hatching. The persistence of MSA-140 on skeletal muscle fibers of the adult rules out the possibility that the antigen is a form of the neural cell adhesion molecule, N-CAM, because N-CAM is lost from muscle fibers except at the neuromuscular synapses in adult chickens (14). Cardiac muscle fibers stained with antibody to MSA-140 had a striated appearance. This distribution suggests that MSA-140 is related to the structure of the myofilament. In the rat ventricle, small bundles of collagen (collagen struts) connect individual cardiac myocytes and are often attached to the myocytes near the region of the Z-bands of the underlying myofilaments (59). Similar intercellular struts have been observed in rat skeletal muscle (7), but neither a distribution coincident with the Z bands nor a difference in the distribution on fast and slow fibers was noted. The vinculin-containing subsarcolemmal-lattice of cardiac myocytes also has a periodicity corresponding to the sarcomeres of the underlying myofibrils (56, 64). MSA-140 could be a component involved in linking the strut structures of the extracellular matrix and the intracellular cytoskeleton of cardiac myocytes.

While many studies have demonstrated that biochemical differences exist between muscle extracts obtained by homogenization of fast and slow muscles (23, 24, 66), few studies at the cellular level have identified sarcolemma constituents that differ among specific fiber types. The results reported here indicate that such differences exist. Immunological analysis of chicken skeletal muscle fibers with a mAb specific for sarcolemmal Na⁺-K⁺ ATPase revealed variability in the immunofluorescence staining intensity among fibers (cf., Fig. 10 in reference 21), and a complex extracellular glycoprotein has been detected primarily at the myotendinous regions of chicken skeletal muscle (13); however, no

correlation between fiber types was noted in either case. The distribution of extracellular components such as laminin, fibronectin, and neuromuscular junction-specific components have not been shown to differ between fast and slow muscle fibers (1, 6, 20, 62). Distinct immunohistochemical staining by mAb's specific for voltage-dependent sodium channel proteins among fast and slow skeletal muscles suggests that differences in sarcolemma-associated proteins may be detected at the cellular level (34).

MSA-slow, MSA-140, and MSA-55 represent three types of antigens primarily, though not exclusively, associated with skeletal muscle that are located on or near the sarcolemma. Not only are there antigens associated with the sarcolemma shared by all fiber types, there are sarcolemma-associated antigens expressed on only fibers of specific types. These antigens distinguish muscle cells from other tissues in the developing limb, distinguish fast from fast/slow or slow muscle fibers, and, because their expression is regulated during development, they may be important in the function of fibers of different physiological types.

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