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Developmental Expression of Spectrins in Rat Skeletal Muscle

Daixing Zhou, Jeanine A. Ursitti, and Robert J. Bloch*

Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201

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Skeletal muscle contains spectrin (or spectrin I) and fodrin (or spectrin II), members of the spectrin supergene family. We used isoform-specific antibodies and cDNA probes to investigate the molecular forms, developmental expression, and subcellular localization of the spectrins in skeletal muscle of the rat. We report that β-spectrin (βI) replaces β-fodrin (βII) at the sarcolemma as skeletal muscle fibers develop. As a result, adult muscle fibers contain only α-fodrin (αII) and the muscle isoform of β-spectrin (βIΣ2). By contrast, other types of cells present in skeletal muscle tissue, including blood vessels and nerves, contain only α- and β-fodrin. During late embryogenesis and early postnatal development, skeletal muscle fibers contain a previously unknown form of spectrin complex, consisting of α-fodrin, β-fodrin, and the muscle isoform of β-spectrin. These complexes associate with the sarcolemma to form linear membrane skeletal structures that otherwise resemble the structures found in the adult. Our results suggest that the spectrin-based membrane skeleton of muscle fibers can exist in three distinct states during development.

INTRODUCTION

The factors that determine the organization and stability of the plasma membranes of eukaryotic cells are poorly understood. Many studies addressing this question have focused on cytoskeletal proteins, such as clathrin and spectrin, that associate with different regions, or domains, at the cytoplasmic surface of the membrane. Spectrin and fodrin are homologous proteins that help to form the membrane-associated cytoskeleton in a wide variety of cells (for reviews, see Bennett and Gilligan, 1993; Hartwig, 1995; and Beck and Nelson, 1996). The spectrins have been extensively studied in erythrocytes, where they play a key role in stabilizing the plasma membrane and generating the biconcave shape typical of these cells (for reviews, see Luna and Hitt, 1992; Delaunay and Dhermy, 1993; and Gallagher and Forget, 1993). Erythrocyte spectrin (also known as spectrin I; Winkelmann and Forget, 1993) is composed of two subunits, α and β, that bind to each other to form dimers and higher oligomers (Morrow and Marchesi, 1981; Morrow *et al.*, 1981; Speicher *et al.*, 1983, 1992; Liu *et al.*, 1984; Ursitti *et al.*, 1991). Fodrin (also known as spectrin II; Winkelmann and Forget, 1993) was initially found in neurons (Levine and Willard, 1981) but is now considered to be the general tissue isoform of spectrin (Bennett and Gilligan, 1993). It, too, is composed of α and β subunits that associate and oligomerize. The oligomeric state and subcellular distributions of the spectrins and fodrins have not been determined for most types of cells, especially in tissues, such as skeletal muscle, that contain both.

Although the α and β subunits of the spectrins and fodrins share high homology, they are encoded by different genes (Huebner *et al.*, 1985; Leto *et al.*, 1988; Hu *et al.*, 1992), the products of which are subject to alternative splicing (Winkelmann *et al.*, 1990; our unpublished results). For example, the 3′ region of the mRNA encoding erythrocyte β-spectrin (βIΣ1) can be alternatively spliced to produce a different C-terminal sequence in skeletal muscle (Winkelmann *et al.*, 1990). The muscle isoform of β-spectrin (βIΣ2) and both the α and β subunits of fodrin (αII and βII, respectively), but not α-spectrin (αI), have been identified in mammalian skeletal muscle by immunological methods

* Corresponding author: Department of Physiology, University of Maryland School of Medicine, 660 West Redwood Street, Baltimore, MD 21201.

(Appleyard *et al.*, 1984; Porter *et al.*, 1990, 1997). Furthermore, avian skeletal muscle in culture undergoes a change in expression of the spectrins, in which one of the β subunits is down-regulated and the other is up-regulated (Nelson and Lazarides, 1983). The identity of these avian subunits is still unclear, however. In mammals, both β subunits are capable of binding to α-fodrin in vitro (Hu and Bennett, 1991) and in vivo (Malchiodi-Albedi *et al.*, 1993; Kennedy *et al.*, 1994; Porter *et al.*, 1997), but how these proteins assemble into a membrane skeleton in skeletal muscle fibers remains an open question.

The spectrin-based membrane skeleton of skeletal muscle has been localized to "costameres," structures that lie on the cytoplasmic surface of the sarcolemma over Z lines and M lines of adjacent sarcomeres (Repasky *et al.*, 1982; Craig and Pardo, 1983; Pardo *et al.*, 1983; Nelson and Lazarides, 1984; Porter *et al.*, 1992). Although their structure and function are still unclear, costameres are believed to link the contractile apparatus of superficial myofibrils to the sarcolemma (Pierobon-Bormioli, 1981; Street, 1983; Shear and Bloch, 1985; Thornell *et al.*, 1985) and may also serve to stabilize the sarcolemma during the contractile cycle. Because the strength of contraction increases greatly after birth, both the expression and organization of spectrin and fodrin in the costameres of skeletal muscle may be developmentally regulated.

Here we report experiments in which we used cDNA probes and isoform-specific antibodies to determine the expression and localization of spectrins in developing skeletal muscle of the rat. Analysis of developmental Northern blots indicates that β-spectrin, unlike β-fodrin, is present in only small amounts in newly formed rat muscle fibers, but that it undergoes a significant increase after birth. As a result, adult muscle fibers contain only α-fodrin and the muscle isoform of β-spectrin. Immunofluorescence and immunoprecipitation experiments show that β-spectrin replaces β-fodrin in the membrane skeleton as muscle fibers develop. During the period in which both β subunits are present in muscle fibers, we find a new form of spectrin complex at the sarcolemma, consisting of α-fodrin together with both β-fodrin and β-spectrin.

MATERIALS AND METHODS

Tissue

Adult female rats and rats with timed pregnancies were purchased from Zivic Miller (Zelienople, PA). To obtain embryonic tissue, mothers were sacrificed under anesthesia with Metofane (Pitman-Moore, Mundelein, IL), the embryos were taken from the uterus, and hindlimb muscle tissue was dissected and frozen immediately in liquid nitrogen. Tissue was stored at −70°C until it was used for RNA isolation or protein extraction.

Generation of Isoform-Specific Antibodies

Monoclonal antibodies to βIΣ1 from human erythrocytes, and rabbit antibodies to the C-terminal sequences of the erythrocyte and muscle isoforms of β-spectrin (βIΣ1 and βIΣ2, respectively, according to the nomenclature of Winkelmann and Forget, 1993), have been described (Porter *et al.*, 1992, 1997). Additional antibodies to β-spectrin (βIΣ*,βIΣ1), α-fodrin (αIIΣ*), and β-fodrin (βIIΣ*) were also generated in rabbits, as described (Bloch and Hall, 1983; Porter *et al.*, 1992, 1997). As the spectrins are highly conserved among mammals (Leto *et al.*, 1988; Hong and Doyle, 1989; Winkelmann *et al.*, 1990; Hu *et al.*, 1992; Ma *et al.*, 1993; Bloom *et al.*, 1993; Chang *et al.*, 1993), we used α-fodrin and β-fodrin from bovine brain and β-spectrin from human erythrocytes to generate antibodies. Briefly, after immunization and repeated boosting, immunoglobulin G (IgG) was isolated from antisera by precipitation with 50% $(NH_4)_2SO_4$, dialyzed against phosphate-buffered saline (10 mM NaP, 145 mM NaCl, pH 7.4), and applied to affinity columns containing purified antigen. The columns were washed with several volumes of buffered saline and then eluted with 50 mM glycine, 500 mM NaCl, pH 2.7. Eluted fractions were collected into tubes containing sufficient 1 M Tris-HCl, pH 8.0, to bring their pH to 7.2. Affinity-purified antibodies were dialyzed against buffered saline containing 10 mM NaN_3 and stored at 4°C.

All antibodies generated against spectrin subunits were cross-adsorbed to eliminate antibodies that reacted with other members of the spectrin superfamily. In some preparations, we applied the affinity-purified antibodies, prepared as above, to affinity columns coupled to one of the other spectrin or fodrin subunits and collected the protein that failed to bind to the column. More recently, we chromatographed the IgG fractions on the inappropriate affinity resin before purifying the specific antibodies on the appropriate resin.

The specificity of the antibodies was assessed initially by enzyme-linked immunoadsorption assays, following the method of Engvall (1980). This revealed strong specificity of each antibody for its appropriate antigen (not shown). Specificity was then examined more rigorously by immunoblotting of proteins separated by SDS-PAGE (Laemmli, 1970). In some cases, samples were electrophoresed for an additional hour after the bromphenol blue tracker dye reached the bottom of the gel, to facilitate identification of individual spectrin and fodrin subunits (Porter *et al.*, 1997). Electrophoretic transfer of peptides from gels to nitrocellulose and immunoblotting of nitrocellulose strips were according to Burnette (1981).

Spectrin was purified from erythrocyte membranes as described (Bennett, 1983), and the β subunit was isolated by chromatography on DEAE-cellulose in the presence of 3 M urea following the method of Yoshino and Marchesi (1984). Fodrin was purified from bovine brain, and the α and β subunits were separated according to Bennett *et al.* (1986). Proteins for immunization or for preparation of affinity matrices were homogeneous by SDS-PAGE analysis. Coupling of proteins to the Sepharose 4B (Pharmacia, Piscataway, NJ) matrix was done after activation of the matrix by cyanogen bromide (March *et al.*, 1974). In some cases, activated matrix was purchased from the manufacturer.

RNA Isolation and Northern Blot Analysis

RNA was isolated from frozen tissue by the guanidinium thiocyanate method described by Chomczynski and Sacchi (1987). Northern blot analysis was performed using the standard glyoxal/dimethyl sulfoxide method. Briefly, total RNA (15 μg) was fractionated in a 1% agarose gel and transferred to a nylon membrane. After fixation by UV light with an UV cross-linker (Stratagene, La Jolla, CA), blots were prehybridized with quick-hybridization solution (Stratagene) for 15 min at 68°C. The ^{32}P-labeled probe (10^6 cpm/ml) was then added and incubation was continued for 2 h. The blots were washed once for 30 min in 2× SSC, 0.1% SDS at room temperature, twice for 15 min in 0.1× SSC, 0.1% SDS at 60°C, and then wrapped in Saran Wrap and exposed to x-ray film

(X-OMAT, Kodak, Rochester, NY). All exposures were tested to ensure that they were in the linear response range of the film. The stringency of hybridization was tested by hybridizing one sample blot in conventional hybridization solution containing 5.5× SSPE, 50% formamide, 5× Denhart's solution, 0.5% SDS, 1 μg/ml Poly A^+, 100 μg/ml salmon sperm DNA at 45°C overnight, followed by washing in 0.1× SSC at room temperature for 30 min and 0.1× SSC at 65°C for an additional 30 min. The results were the same using both solutions, but the quick-hybridization solution yielded lower backgrounds.

Radioactive probes were generated using a random-labeling kit from Life Technologies-BRL (Gaithersburg, MD). The probes used were: β11 (Bloom *et al.*, 1993), containing repeats 2–11 of mouse β-spectrin cDNA, kindly provided by Dr. M. Bloom (National Institutes of Health, Bethesda, MD); B12–1 (Ma *et al.*, 1993), the C-terminal of mouse β-fodrin cDNA, a generous gift from Dr. W. Zimmer (University of South Alabama College of Medicine, Mobile, AL); α4A, containing repeats 11 to the C terminus of rat α-fodrin cDNA (our unpublished results); pHαSp6 (Curtis *et al.*, 1985), containing repeats 14–17 of human α-spectrin cDNA, purchased from American Type Culture Collection (Rockville, MD); β2A (Winkelmann *et al.*, 1990), the 3′ region of human muscle spectrin (βIΣ2), kindly provided by Dr. J. Winkelmann (University of Cincinnati, Cincinnati, OH), which was digested with *Sma*I to release the muscle-specific region. The probe for the erythrocyte-specific C terminus was generated by 3′ RACE (rapid amplification of cDNA ends) polymerase chain reaction (Ursitti and Bloch, unpublished data). The hybridized blots were exposed to films for 3 d. Blots were then stripped and hybridized to ^{32}P-labeled 18S rRNA oligomers, kindly provided by Dr. R. Wade (University of Maryland School of Medicine, Baltimore, MD), to normalize for the amount of RNA loaded into each lane of the gel. Because the 18S rRNA signal was very strong, the blots probed for this marker were only exposed for 1 h. The intensity of the signal was quantified using the ImageQuant program (Molecular Dynamics, Sunnyvale, CA) after scanning the x-ray film with a densitometer, or with a Molecular Dynamics Phosphoimager. The normalized values were generated by first adjusting the value of each sample for the RNA load and then dividing by the adult value.

Analysis of Spectrins in Muscle Tissue

Frozen tissue was homogenized in a Virtis "45" homogenizer (Virtis, Gardenier, NY) on ice for 2 min (4 × 30 s) in buffer containing 1% deoxycholate, 1% Nonidet P-40, 10 mM sodium phosphate, 0.5 M NaCl, 2 mM EDTA, pH 6.8 (Hoffman *et al.*, 1989), supplemented with protease inhibitors (0.15 mM phenylmethylsulfonyl fluoride, 0.22 U/ml aprotinin, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml antipain, 200 μg/ml soybean trypsin inhibitor). The homogenized sample was then centrifuged at 16,000 rpm in a Sorvall SS-34 rotor (Dupont-Sorvall, Wilmington, DE) to remove insoluble material. Aliquots of the supernatant containing 50 μg of protein were boiled in sample buffer (Laemmli, 1970), electrophoresed, and transferred to nitrocellulose membranes (Burnette, 1981). After blocking in phosphate-buffered saline containing 3% (wt/vol) nonfat dry milk solids and 0.1% Triton X-100 for 2 h, strips of nitrocellulose were incubated with one of the anti-spectrin antibodies for 2 h, washed, and incubated for 1 h with secondary antibody conjugated to alkaline phosphatase. Dilutions of secondary antibodies were 1:10,000 for anti-α-fodrin and anti-β-spectrin, and 1:1,000 for anti-β-fodrin. The chromogenic reaction to detect bound antibody was carried out using a BCIP/NBT kit from Kirkegaard and Perry (Gaithersburg, MD). All reactions were performed at room temperature.

Immunoprecipitation

Homogenates of hindlimb skeletal muscle were prepared as described above from postnatal day 1 (P1) and adult rats that had been perfused through the left ventricle with ice-cold buffered saline supplemented with protease inhibitors. To clear the muscle homogenate before immunoprecipitation, 1 mg of each homogenate was incubated overnight at 4°C with 100 μl of a suspension of Trisacryl beads covalently linked to protein A (Pierce Chemical, Rockford, IL). Antibodies for immunoprecipitation were bound overnight at 4°C to a second aliquot of beads by mixing 10 μg of either rabbit anti-α-fodrin or anti-β-fodrin antibody with 50 μl of the bead suspension. The precleared muscle homogenates from P1 or adult muscle were mixed with each antibody–bead complex for 4 h at 4°C. The supernatant was removed and the beads were washed four times with buffered saline containing 0.5% Tween 20 before elution by boiling in 60 μl sample buffer (Laemmli, 1970). The samples (30 μl per 12-mm lane) were electrophoresed on 5% acrylamide gels and visualized by silver staining. Alternatively, the electrophoresed samples were transferred to nitrocellulose membrane and probed with antibodies to β-spectrin, α-fodrin, and β-fodrin to determine the identity of the precipitated products (data not shown).

Immunofluorescence

Anesthetized rats were perfused through the left ventricle with buffered saline followed by 2% paraformaldehyde in buffered saline, to fix muscle in situ. Diaphragm or sternomastoid muscle from postnatal day 7 (P7) and adult rats, and in some cases also hindlimb muscle, was removed and plunged into a slush of liquid nitrogen. For studies of embryonic tissue, fetuses were removed from the anesthetized mother and placed on ice. Diaphragm or hindlimb muscle from the embryo was wrapped in a piece of diaphragm from the mother and frozen in a slush of liquid nitrogen. Sections were prepared on a cryostat (Reichert-Jung, Cambridge Instruments, Deerfield, IL) at thicknesses of 5 μm for adult tissue or 12 μm for embryonic tissue. Frozen sections were collected on slides pretreated with chrom-alum gelatin and stored at −70°C.

For immunolabeling, samples were pretreated for 10 min in buffered saline containing 1 mg/ml bovine serum albumin and then incubated for 1 h with primary antibodies, diluted in the same solution. All rabbit antibodies were used at 2 μg/ml; 4C3 monoclonal anti-β-spectrin was used at 25 μg/ml, and monoclonal antibody to desmin (Boehringer-Mannheim, Indianapolis, IN) was used at 4 μg/ml. Nonimmune rabbit IgG (5 μg/ml) combined with anti-desmin or MOPC 21 mouse IgG (5 μg/ml) was used as a control in every experiment. After extensive washing, slides were counterstained for 1 h with fluoresceinated goat anti-mouse IgG (FGAM, 10 μg/ml) and tetramethylrhodaminylated goat anti-rabbit IgG (RGAR, 10 μg/ml), both from Jackson Immunoresearch (West Grove, PA). All incubations were carried out at room temperature. Samples were washed extensively, mounted in a solution containing 9 parts glycerol, 1 part 1 M Tris-HCl, pH 8.0, supplemented with 1 mg/ml *p*-phenylenediamine, to reduce photobleaching (Johnson *et al.*, 1982).

Samples were first viewed under conventional epifluorescence optics and then under confocal optics, using a Zeiss 410 confocal laser scanning microscope (Carl Zeiss, Tarrytown, NY). Images were obtained at maximum resolution, sharpened using the MetaMorph image-processing program (Universal Imaging, West Chester, PA), and printed on a Codonics NP-1600 photographic network printer (Codonics, Middleburg Heights, OH).

Materials

Unless otherwise indicated, all materials were purchased from Sigma Chemical (St. Louis, MO) and were the highest grade available.

RESULTS

The aim of our experiments was to determine the molecular characteristics of the spectrin-based mem-

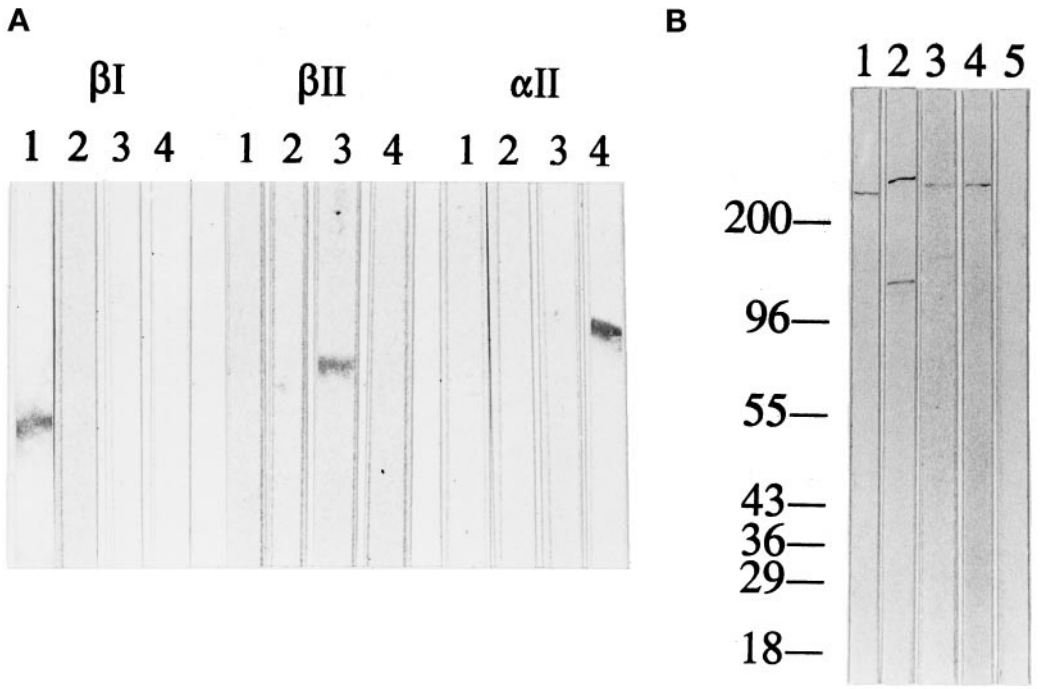

Figure 1. Specificity of anti-spectrin antibodies determined by immunoblotting. Purified proteins (A) or tissue extracts (B) were boiled in sample buffer, subjected to SDS-PAGE in gels containing 5% acrylamide (A) or 5–15% acrylamide gradients (B), transferred to nitrocellulose, and subjected to immunoblotting with antibodies generated against β-spectrin (βIΣ*), β-fodrin (βIIΣ*), and α-fodrin (αIIΣ*) that were affinity purified and cross-adsorbed (see MATERIALS AND METHODS). (A) Lanes 1–4 contain purified β-spectrin, α-spectrin, β-fodrin, and α-fodrin, respectively. (B) Immunoblots of rat muscle or hippocampal (lane 4 only) extracts were incubated with antibodies to β-spectrin (lane 1), α-fodrin (lane 2), β-fodrin (lanes 3 and 4), or to a control rabbit IgG (lane 5). Anti-β-spectrin recognizes a single band or a closely spaced doublet at ~265 kDa. Anti-α-fodrin recognizes a single band at ~280 kDa and a well-characterized proteolytic fragment (Harris *et al.*, 1988) at ~150 kDa. Anti-β-fodrin reacts faintly with bands at ~270 and ~170 kDa in extracts of skeletal muscle (lane 3) but reacts strongly with a single band at ~270 kDa in hippocampal extracts (lane 4). The results indicate that each of the antibodies reacts specifically with its antigen and not with other proteins in tissue extracts or with the other spectrins assayed.

brane skeleton of skeletal muscle and to learn whether these characteristics change with development. Two sets of reagents were essential: cDNA probes that could distinguish the mRNAs encoding the different spectrins and fodrins, and antibodies that could detect the proteins in muscle tissue. The cDNA probes are described in MATERIALS AND METHODS. We had already prepared some isoform-specific anti-spectrin antibodies in rabbits and mice and demonstrated their specificities (Porter *et al.*, 1997). For the present project, we prepared additional antibodies and checked their specificities, first by enzyme-linked immunoadsorption assays (data not shown) and then by immunoblotting (Figure 1A). Our results show that the antibodies to human β-spectrin (βIΣ*), bovine β-fodrin (βIIΣ*) and α-fodrin (αIIΣ*) are specific for their respective antigens. These antibodies also recognize proteins of the appropriate molecular masses in immunoblots of extracts of rat skeletal muscle (Figure 1B), indicating that they are specific for the appropriate subunits of spectrin and fodrin from the rat. Labeling by anti-β-fodrin was difficult to detect, however, and it was invariably accompanied by labeling of an additional band at ~170 kDa (Figure 1B, lane 3). Our subsequent experiments showed that adult rat skeletal muscle contains little β-fodrin (see below). The smaller band is likely to be a breakdown product of β-fodrin, perhaps similar to the well-characterized 150 kDa fragment of α-fodrin (visible in Figure 1B, lane 2; see Harris *et al.*, 1988), or an alternatively spliced product of the β-fodrin gene that has not yet been identified. We tested the specificity of the anti-β-fodrin antibodies further in immunoblots of rat hippocampal extracts (Figure 1B, lane 4), which contain significant amounts of α-fodrin, β-fodrin, and β-spectrin (our unpublished results). In this case, anti-β-fodrin recognized a single band at the appropriate molecular weight. Our results, therefore, clearly establish the specificity of anti-β-fodrin, anti-α-fodrin, and anti-β-spectrin for their respective antigens in excitable tissues. We applied these reagents to the study of the spectrins in skeletal muscle of embryonic, neonatal, and adult rats.

Northern Blot Analysis

We used specific cDNA probes to probe blots of total RNA prepared from embryonic, neonatal, and adult muscle tissue to determine when during development the spectrins are expressed (Figure 2). In Northern blots of mRNA prepared from developing skeletal muscle, we were able to resolve only two transcripts encoding the fodrins, one for α-fodrin (Figure 2C) and another for β-fodrin (Figure 2B). Expression of α-fodrin increased significantly with age (Figure 2C′),

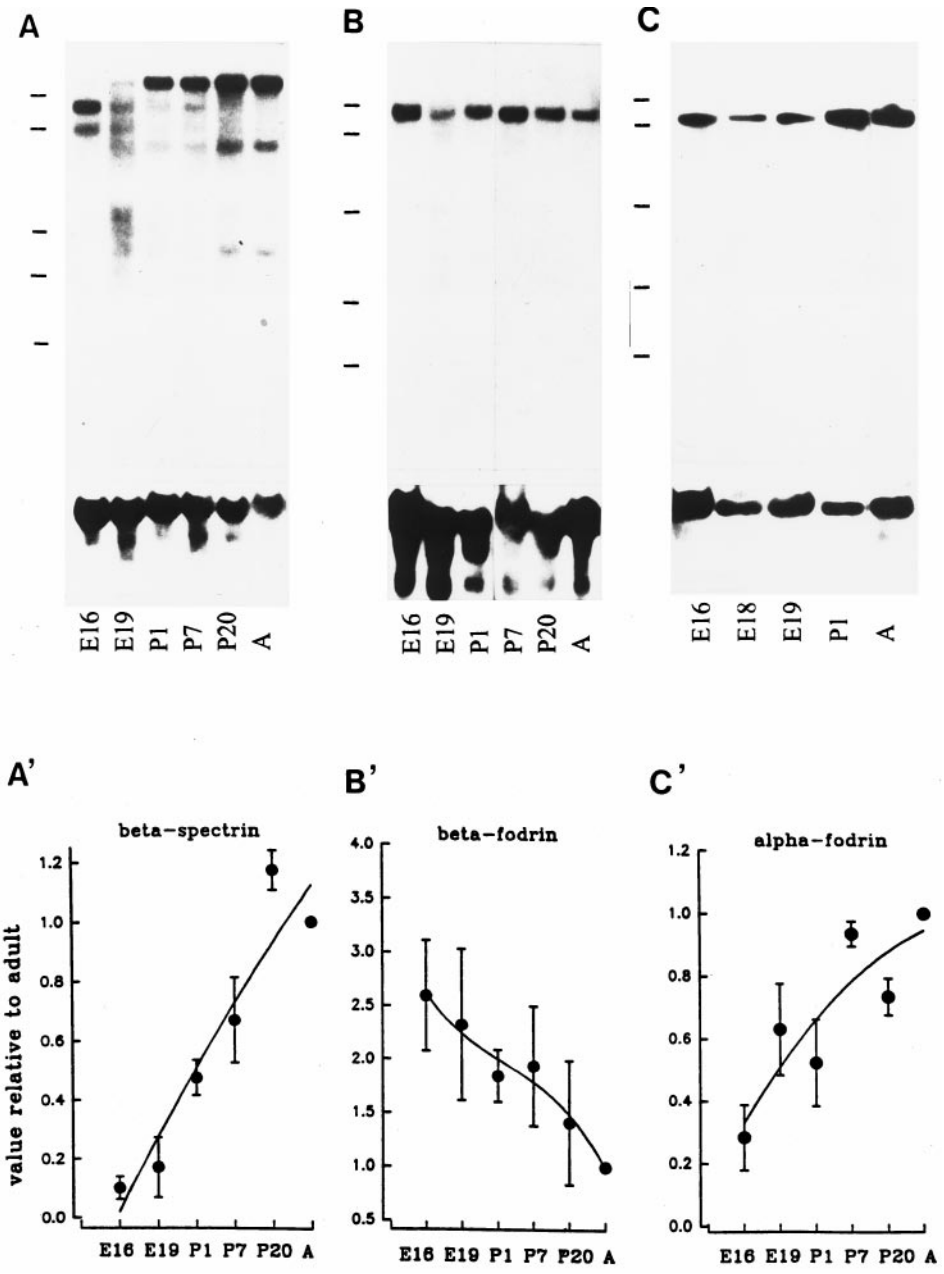

Figure 2. Developmental expression of β-spectrin, α-fodrin, and β-fodrin in rat skeletal muscle analyzed by Northern blot. RNAs were isolated from rat hindlimb muscle at different ages, fractionated on 1% agarose gels, and transferred to nylon membranes. Three different membranes were hybridized to cDNA probes specific for β-spectrin (A), β-fodrin (B), and α-fodrin (C). The membranes were then stripped and hybridized with probes to 18S rRNA to indicate the relative amounts of RNA loaded (shown at the bottom of each blot). After normalization against the rRNA signal, the level of expression of each message at each age was plotted relative to its corresponding adult value: A′, β-spectrin (only the 11-kb transcript is shown); B′, β-fodrin; C′, α-fodrin. The values were averages of at least three independent experiments; values for the mean ± SEM molecular size standards, indicated to the left of each blot, are (from top to bottom) 9.5, 7.5, 4.4, 2.4, and 1.4 kb. The developmental stages tested were E16, E18, and E19 (embryonic days 16, 18, and 19), P1 (postnatal day 1), and adult (A).

whereas the relative expression of β-fodrin decreased significantly between embryonic day 16 (E16) and adulthood (Figure 2B′). We could not detect a transcript for α-spectrin in skeletal muscle at any developmental stage (not shown), in agreement with previous results (Appleyard *et al.*, 1984; Porter *et al.*, 1992).

The expression of β-spectrin in developing skeletal muscle tissue was considerably more complex (Figure 2A). We detected at least five alternatively spliced transcripts with β11 as the probe (see MATERIALS AND METHODS) for β-spectrin, at 3.5, 6.0, 7.5, 9.0, and 11.0 kilobases (kb), in agreement with results reported by Bloom *et al.* (1993). Furthermore, the relative amounts of these transcripts changed with development (see Figure 2A; Figure 2A′ shows changes of the 11-kb transcript only). These results indicated that the transcripts at 3.5, 6.0, and 11.0 kb all increased with age, while the 7.5- and 9.0-kb transcripts decreased, reaching levels that were almost undetectable in total RNA preparations after P7. In E16 muscle, by contrast, the 7.5- and 9.0-kb transcripts predominated (Figure 2A), and only small amounts of the 11.0-kb transcript were detectable (Figure 2A′). To determine whether the different transcripts encode different C-

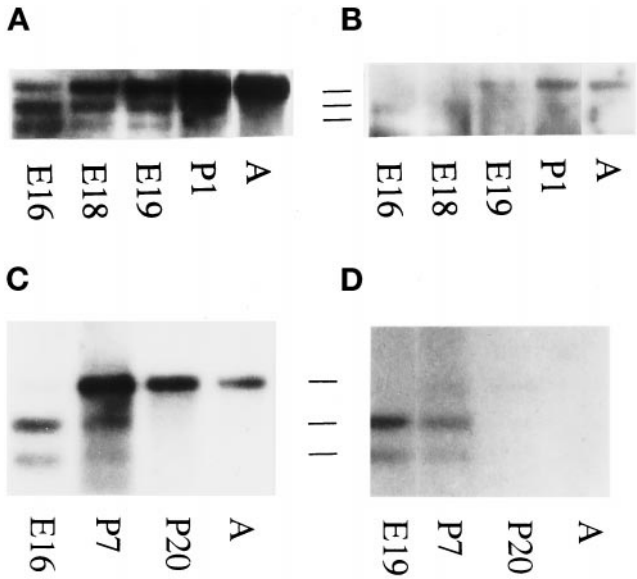

Figure 3. Isoforms of β-spectrin encoded by the 7.5-, 9.0-, and 11-kb transcripts. Two RNA blots were hybridized to a probe shared by both erythroid and skeletal muscle β-spectrin cDNA (panels A and C) and show three bands at 11, 9.0, and 7.5 kb. The membranes were then stripped and rehybridized with probes for muscle-specific (βIΣ2, panel B) and erythrocyte-specific (βIΣ1, panel D) 3′ sequences. The results indicate that the predominant 11-kb transcript in postnatal muscle is muscle specific, while the 7.5- and 9.0-kb transcripts encode the alternatively spliced form found in erythrocytes.

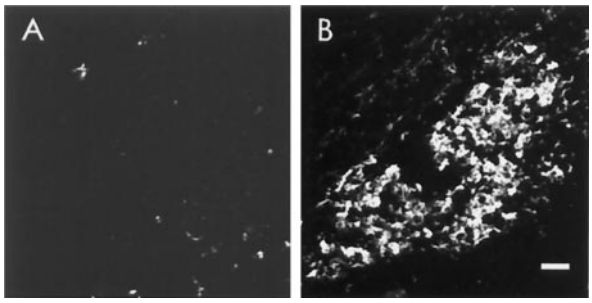

Figure 4. Absence of erythrocyte β-spectrin in E16 muscle fibers. Cryosections of E16 rat hindlimb were double-labeled with polyclonal antibodies to the extreme C-terminal sequence of erythroid β-spectrin (βIΣ1, panel A) and monoclonal antibodies to desmin (panel B), to identify the muscle cells, followed by rhodamine-conjugated anti-rabbit and FITC-conjugated anti-mouse secondary antibodies. The result shows that the desmin-positive muscle cells seen in E16 hindlimb do not contain the erythrocyte isoform of β-spectrin. Bar, 20 μm.

terminal, alternatively spliced forms of β-spectrin, we probed Northern blots with cDNA sequences that distinguish between the erythrocyte and muscle isoforms (Winkelmann *et al.*, 1990). Using a sequence specific for the muscle isoform, we detected only the 11.0-kb transcript (Figure 3, A and B). This transcript increased dramatically with development. The probe specific for the C-terminal sequence of the erythrocyte form hybridized to both the 7.5- and 9.0-kb transcripts in embryonic muscle, but not to any of the transcripts in adult muscle (Figure 3, C and D). This is most readily explained by the decrease in hematopoiesis that occurs in late embryogenic and early postnatal life and by the fact that we used perfused muscle to prepare all samples from rats older than P7. Thus, the 7.5-kb and 9.0-kb transcripts are probably due to the presence of reticulocytes in the circulation of embryonic muscle samples.

Immunolocalization of Spectrins in Muscle

Embryonic Muscle We confirmed that erythrocyte β-spectrin was present only in the circulation of embryonic muscle using antibodies specific for the C-terminal sequence of this alternatively spliced form. In frozen sections of embryonic rat hindlimb muscle, these antibodies failed to label muscle fibers, which did, however, label brightly with antidesmin (Figure 4). The labeling we observed with the anti-β-spectrin specific for the erythrocyte C-terminal sequence was limited to the capillaries located between the myofibers and in surrounding tissue. In frozen sections of E16 diaphragm muscle, we also found no labeling of muscle fibers by this antibody, but nearby liver cells were labeled (not shown), consistent with hematopoiesis occurring in embryonic liver (Russell and Bernstein, 1968).

Although embryonic muscle fibers did not contain β-spectrin with the erythrocyte C-terminal sequence (βIΣ1), they did contain β-spectrin. In addition to labeling circulating red cells, polyclonal, rabbit antibodies specific for β-spectrin labeled the sarcolemma of muscle fibers in the diaphragms of E16 rats (Figure 5A). In thick sections examined by confocal laser scanning microscopy, labeling was present in strands that were usually oriented perpendicular to the longitudinal axis of the muscle fibers (see Figure 6A′). These results suggest that a membrane skeleton containing β-spectrin is already present in E16 rat muscle. The fact that the antibody specifically recognizing the erythrocyte-specific C terminus of β-spectrin (βIΣ1) failed to label the sarcolemma suggested that this β-spectrin is the muscle-specific isoform (βIΣ2). This

Figure 5 (facing page). Presence of β-spectrin, α-fodrin, and β-fodrin at the sarcolemma of embryonic rat skeletal muscle. Cross-sections of E16 (A–C, A′-C′) and E19 (D–F, D′–F′) rat diaphragm were labeled with isoform-specific polyclonal antibodies to β-spectrin (A and D), α-fodrin (B and E), and β-fodrin (C and F) along with monoclonal antibodies to desmin (A′–F′). The results show that all three subunits are expressed in embryonic rat skeletal muscle cells, where they assemble at or near the sarcolemma. Bar, 20 μm.

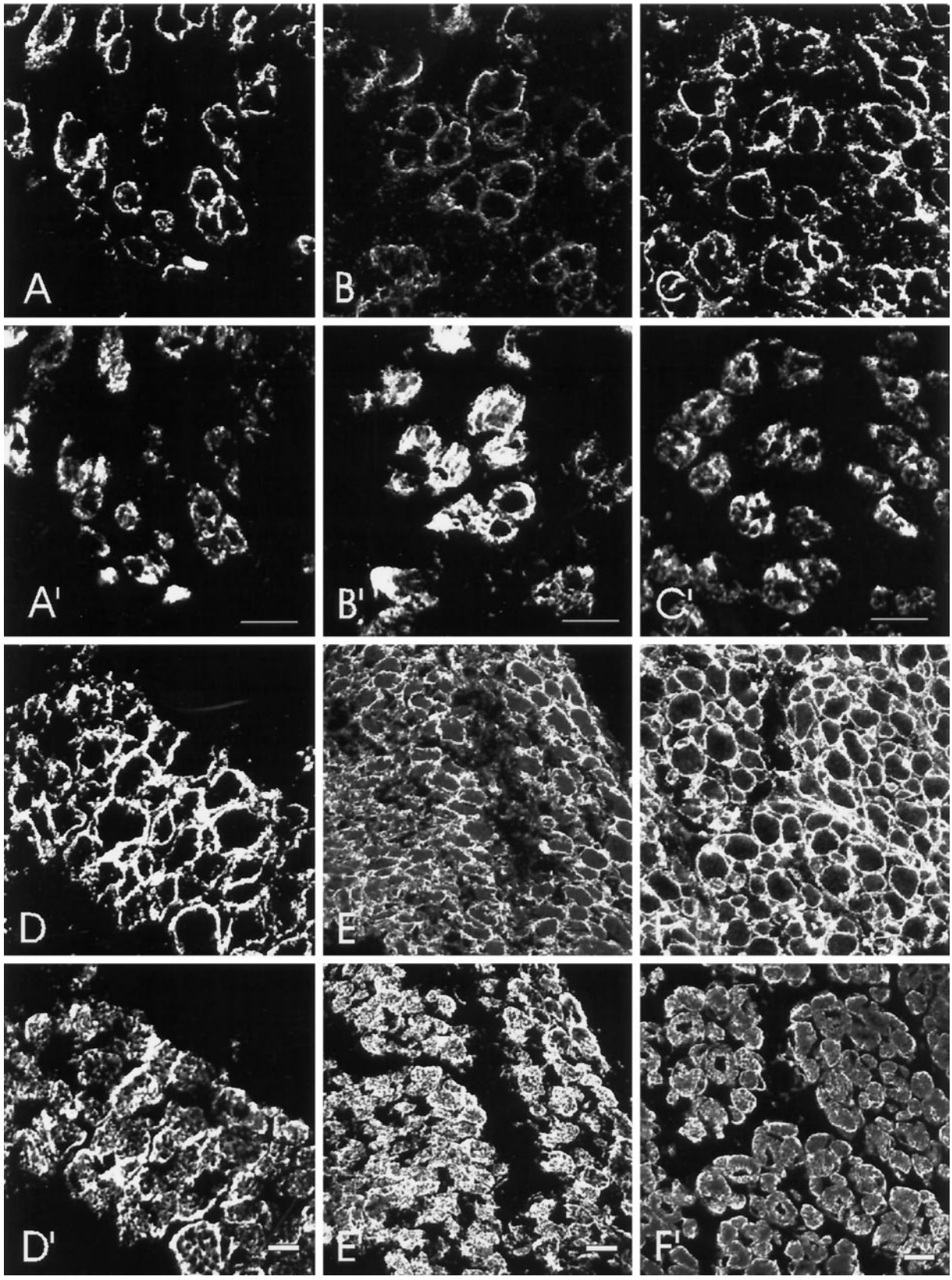

Figure 5.

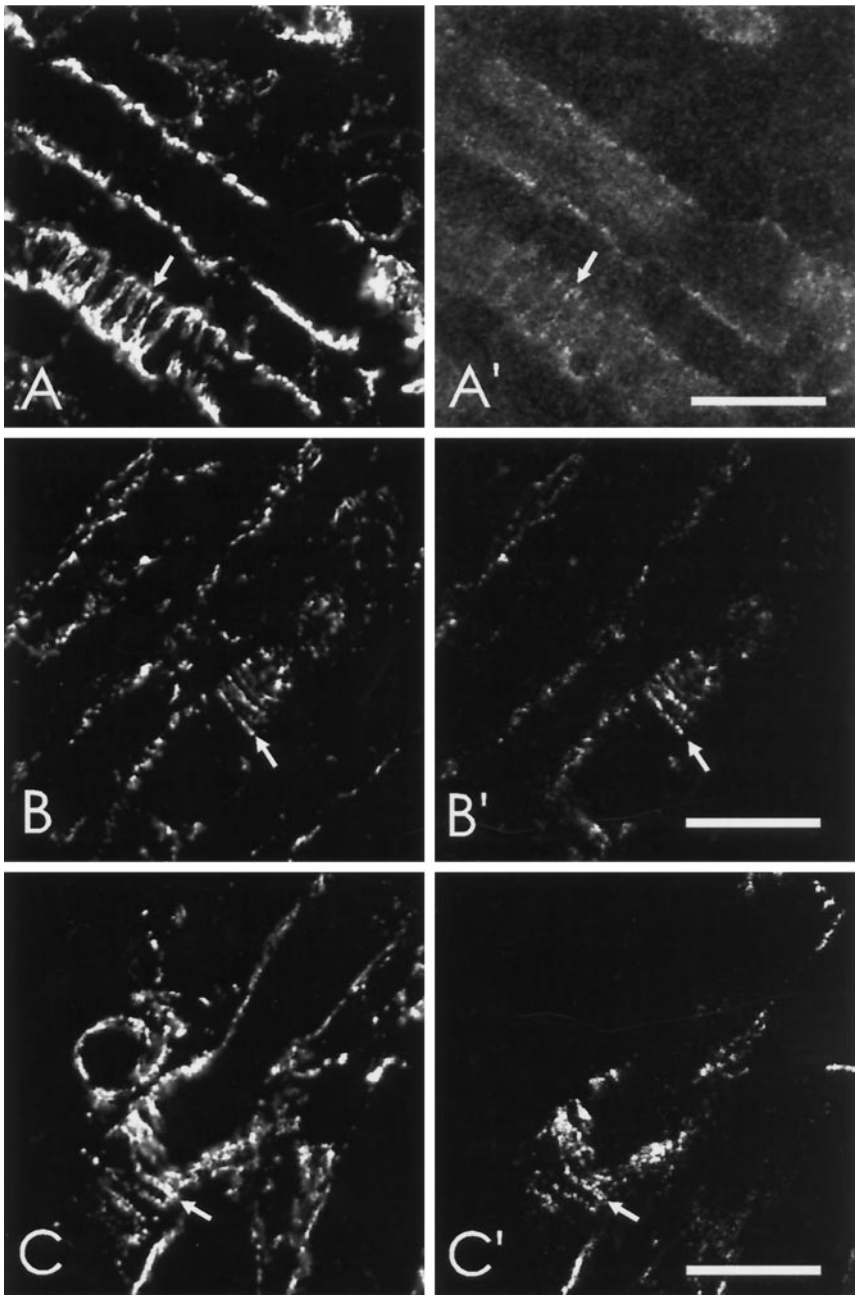

Figure 6. β-Fodrin and α-fodrin codistribute with β-spectrin at the sarcolemma of embryonic skeletal muscle. Longitudinal sections of E16 (A and A′) or E19 (B, B′, C, and C′) diaphragm were double-labeled with polyclonal antibodies to α-fodrin (A and B) or β-fodrin (C) and 4C3 monoclonal antibodies to β-spectrin (A′–C′). Each pair of labeled subunits codistributes in strands at the sarcolemma of embryonic muscle fibers (arrows). Bar, 20 μm.

observation has recently been confirmed in the mouse through the use of antibodies specific for the C-terminal alternatively spliced sequence of βIΣ2 (Weed, 1996).

We also used isoform-specific antibodies to learn whether α- and β-fodrin are present in the muscle fibers of embryonic rat muscle. Affinity-purified and cross-absorbed antibodies to each of these subunits recognized structures in embryonic diaphragm muscle that resembled those seen with antibodies to β-spectrin (Figure 5, B and C). These antibodies also labeled other tissues in muscle, including blood vessels, nerve, and connective tissue.

We obtained similar results in immunofluorescence studies of diaphragm muscle from E19 rats. As expected (Harris, 1981), muscle fibers in 19-d-old embryos were fasciculated. Antibodies to β-spectrin, α-fodrin, and β-fodrin all labeled the sarcolemma of muscle fibers (Figure 5, D–F). In addition, anti-β-spectrin labeled erythrocytes in the capillary bed, while the antibodies to fodrins labeled connective tissue, blood vessels, and nerves.

As in E16 muscle, the sarcolemma of E19 muscle could be labeled with anti-spectrin antibodies to reveal structures oriented perpendicular to the long axis of the fibers. At E19, however, many more of these structures were present, and they were organized more extensively (see Figure 6). In addition, they seemed to form complete rings around individual muscle fibers that defined the cylindrical shape of the fibers when reconstructed from a series of optical sections (not shown).

The presence in embryonic muscle fibers of both β-spectrin and β-fodrin, together with α-fodrin, raised the question of whether these three proteins were present in the same structures. We performed double immunofluorescence experiments to address this question. Frozen sections of muscle from E19 rats were labeled simultaneously with 4C3, a monoclonal antibody to β-spectrin (Porter *et al.*, 1997), and with rabbit antibodies either against α-fodrin or β-fodrin (see MATERIALS AND METHODS). The bound antibodies were detected with fluoresceinated and tetramethylrhodaminylated secondary antibodies specific for mouse and rabbit IgGs, respectively. In agreement with the results of Northern blot analysis, the monoclonal antibodies to β-spectrin labeled the sarcolemma of E16 skeletal muscle only faintly (Figure 6B), while α-fodrin was easily detectable in the same regions (Figure 6A). Comparison of the labeling patterns of monoclonal anti-β-spectrin to polyclonal antibodies to the fodrin subunits in E19 skeletal muscle also revealed significant overlap, as well as some differences (Figure 6, C–F). The latter are probably due to differences in intensity of labeling obtained with the monoclonal and polyclonal antibodies, as we observed similar disparities when we compared monoclonal and polyclonal antibodies to β-spectrin (not shown). These differences are therefore probably not significant. Nevertheless, the overlap of labeling that we did observe strongly suggests that the three subunits, β-spectrin, β-fodrin, and α-fodrin, assemble into many of the same or closely associated structures in embryonic muscle.

Postnatal Day 7 Muscle Except for their smaller size, muscle fibers at P7 appear to be very similar to those of adult muscle. β-Spectrin was located at the sarcolemma and was also concentrated at the neuromuscular junction (Figure 7A). In longitudinal sections, β-spectrin often showed a punctate labeling pattern along the sarcolemma (Figure 7A′). Three-dimensional reconstructions of serial optical sections revealed this labeling to be present in a costameric pattern (data not shown).

α-Fodrin was also localized at the sarcolemma (Figure 7B) and had the same kind of punctate labeling pattern as seen in longitudinal sections (Figure 7B′), suggesting that α-fodrin was also assembled into costameres at this age. In addition, rabbit anti-α-fodrin labeled connective tissue and nerves, as well as capillaries, which were especially brightly labeled. By contrast, anti-β-fodrin labeled the capillaries, nerve, and connective tissue, but it barely labeled the sarcolemma (Figure 7C). Only careful examination revealed some residual β-fodrin at the sarcolemma in limited regions of the muscle, whether observed in cross- or longitudinal sections (Figure 7C′, arrow).

Adult Muscle β-Spectrin in adult skeletal muscle was present at the sarcolemma (Figure 7D) in a costameric pattern (Figure 7D′). As previously reported (Porter *et al.*, 1992), labeling for β-spectrin overlies the M line and Z line in many myofibers (not shown), but in others the labeling over the Z line could be seen to split into two strands (Figure 7D′). β-Spectrin was also present in strands parallel to the long axis of the muscle fiber, as reported (Porter *et al.*, 1992).

α-Fodrin at the sarcolemma of adult muscle fibers was also costameric but in a pattern distinct from that of β-spectrin (Figure 7E′; see also Porter *et al.*, 1997). In particular, α-fodrin was detected primarily over the Z line, where it was never observed to split, and only infrequently over the M line and in the longitudinal strands commonly seen in samples labeled with antibodies to β-spectrin (Figure 7E′). In addition, α-fodrin was found in capillaries, nerves, and connective tissue (Figure 7E).

Like α-fodrin, β-fodrin was present in capillaries, connective tissue, and nerves (data not shown), but it could not be detected at the sarcolemma of muscle fibers (Figure 7, F and F′). To confirm that skeletal muscle fibers contain β-spectrin but no β-fodrin, frozen sections were double labeled with monoclonal antibodies to β-spectrin and polyclonal antibodies to β-fodrin. Although the sarcolemma was brightly labeled with anti-β-spectrin, labeling for β-fodrin was not detectable near the sarcolemma, except at sites that could be accounted for by capillaries, nerves, or connective tissue (Figure 8). This was also true of the neuromuscular junction (Figure 8, arrowheads), where labeling by anti-β-spectrin was enhanced (e.g., Bloch and Morrow, 1989; Bewick *et al.*, 1996), perhaps due to the presence of postsynaptic folds. As the same antibody to β-fodrin labeled the embryonic muscle cells well, we conclude that adult skeletal muscle fibers contain no detectable β-fodrin. A similar conclusion has been reached recently by Weed (1996) in mouse skeletal muscle.

Immunoprecipitations

Our immunofluorescence results suggest that the membrane skeleton of embryonic and early postnatal muscle fibers contains α-fodrin, β-fodrin, and muscle β-spectrin, whereas that of adult muscle fibers contains only α-fodrin and muscle β-spectrin. To determine whether the composition of spectrin complexes

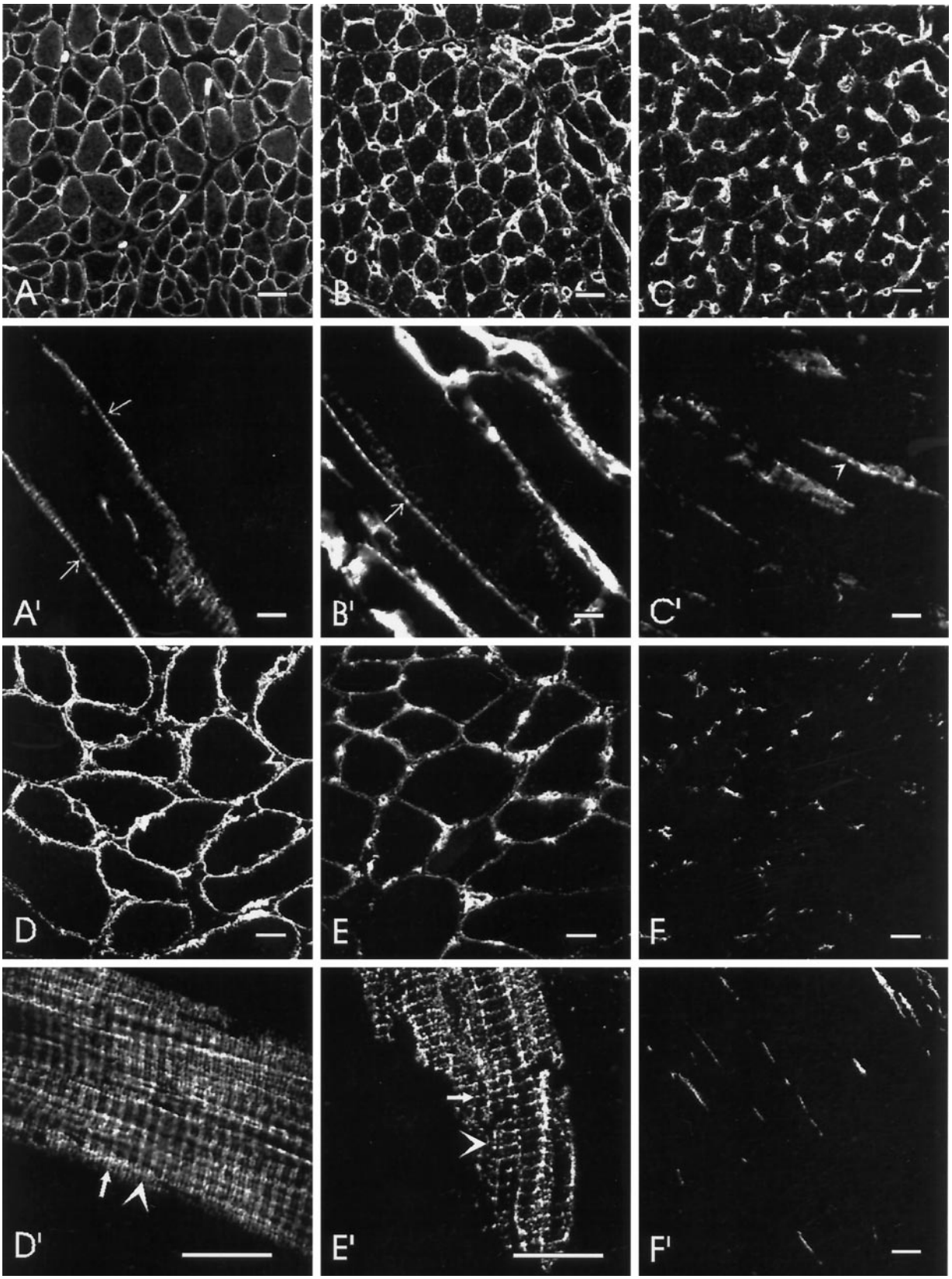

Figure 7.

changes with development, we immunoprecipitated the spectrin complexes from homogenates of hindlimb muscles of newborn and adult rats with either anti-α-fodrin or anti-β-fodrin. (Immunoprecipitation with anti-β-spectrin produced results consistent with those obtained with antifodrins but it was less efficient; data not shown.) Both antifodrin antibodies precipitated muscle β-spectrin together with α- and β-fodrin from samples of P1 muscle (Figure 9, lanes 1 and 2). The fact that antibodies to β-fodrin precipitated β-spectrin as well as α-fodrin suggests that the two β-subunits form part of a single complex. Immunoprecipitation with anti-α-fodrin confirms the identity of these subunits. (We expect this antibody to precipitate all three subunits, as α-fodrin can form heterodimers with both β-fodrin and β-spectrin in muscle tissue; Porter *et al.*, 1997.) This is consistent with our immunofluorescence data that indicate that these three subunits assemble together to form the membrane skeleton of late embryonic and early postnatal muscle fibers. By contrast, immunoprecipitation from homogenates of adult muscle yielded the same three subunits only when anti-α-fodrin was used; immunoprecipitation with anti-β-fodrin yielded only α- and β-fodrins. The fact that antibodies to β-fodrin failed to precipitate β-spectrin suggests that the complex consisting of the two β-subunits and α-fodrin, which is present in neonatal muscle, is absent in the adult. This too is consistent with our immunofluorescence data showing that, in adult skeletal muscle tissue, α- and β-fodrin are present together only in nonmuscle cells, whereas α-fodrin is present together with β-spectrin in muscle fibers, which lack β-fodrin. Immunoprecipitation therefore confirms our immunofluorescence results and extends them by demonstrating directly the presence of heteromeric complexes of α-fodrin, β-fodrin, and β-spectrin in developing muscle fibers.

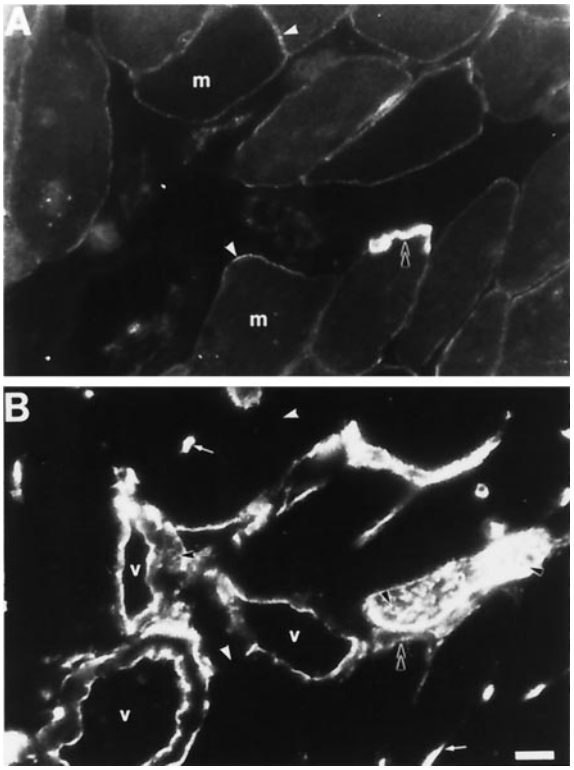

Figure 8. β-Fodrin and β-spectrin in adult rat skeletal muscle. Cross-sections of adult rat diaphragm were double-labeled with monoclonal antibodies to β-spectrin (A) and polyclonal antibodies to β-fodrin (B). Although anti-β-fodrin antibodies label the blood vessels (v) and nerves (n) brightly, β-fodrin was not detected at the sarcolemma of skeletal myofibers (white arrowheads). β-Spectrin was found at the sarcolemma (A, white arrowheads) and was particularly concentrated at the neuromuscular junction (B, double black arrowhead). Capillaries are indicated with small white arrows; β-fodrin at the membrane of a cell in the blood vessel wall is indicated with a small black arrow. Nerve fibers are indicated with single black arrowheads. m, muscle fiber; v, blood vessel. Bar, 20 μm.

Figure 7 (facing page). Spectrins in P7 and adult rat skeletal muscle. Cross-sections (A–F) and longitudinal sections (A′–F′) of 7-d-old (A–C, A′–C′) and adult (D–F, D′–F′) rat diaphragms were labeled with polyclonal antibodies to β-spectrin (A, A′, D, and D′), α-fodrin (B, B′, E, and E′), and β-fodrin (C, C′, F, and F′). The labeling of β-fodrin seen in C and C′ is mostly in capillaries, which are also labeled by anti-α-fodrin (B and B′), but not by anti-β-spectrin. Labeling of the sarcolemma by anti-β-fodrin at P7 is barely detectable. However, some residual punctate labeling could still be seen in longitudinal sections (e.g., arrowheads in C′). By contrast, β-spectrin and α-fodrin are readily apparent at the sarcolemma of P7 muscle, where they are present at punctate structures typical of costameres (e.g., arrows in A′ and B′). β-Spectrin is seen over both the Z lines and M lines in adult muscle (D′, Z line indicated by arrow; M line indicated by arrowhead), with the labeling pattern split into two lines over the Z line. α-Fodrin also localizes over the Z line, but does not show a split there; it is present at M lines to a much lesser extent (E′). Note also that labeling by anti-β-spectrin but not by anti-α-fodrin is present in longitudinally oriented structures, as previously reported (Porter *et al.*, 1997). Bars: A–F, D′–F′, 20 μm; A′–C′, 10 μm.

DISCUSSION

The composition, organization, and mechanisms of assembly of spectrin-based membrane skeletons in cells other than the erythrocyte are still poorly understood, for several reasons. One problem has been the availability of antibodies specific for each of the spectrins and for the alternatively spliced forms of these proteins that have recently been identified. An additional problem has been that of finding a cell like the erythrocyte in which large areas of the plasma membrane are organized similarly and in which the spectrins are accessible to both biochemical and morphological studies. To address the first problem, we generated specific antibodies by repeated immunization, extensive cross-adsorption and affinity purification, and by preparing peptide-specific antibodies that distinguish between the two known alternatively spliced forms of β-spectrin. Our interest in skeletal

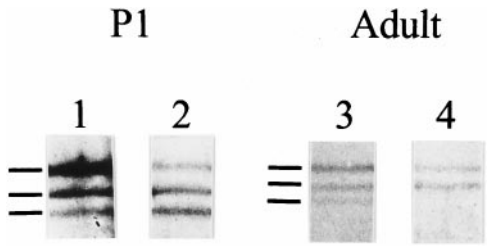

Figure 9. Immunoprecipitation of spectrin and fodrin from skeletal muscle using anti-fodrin antibodies. Spectrin and fodrin were immunoprecipitated from homogenates of P1 (lanes 1 and 2) and adult skeletal muscle (lanes 3 and 4) using anti-α-fodrin (lanes 1 and 3) or anti-β-fodrin (lanes 2 and 4) antibodies. The immunoprecipitated products were electrophoresed on 5% acrylamide gels and visualized by silver staining. The immunoprecipitated proteins are marked by lines to the left of each set. From top to bottom, they are: α-fodrin, β-fodrin, and muscle β-spectrin.

muscle addressed the second problem. Much of the sarcolemma of skeletal muscle fibers is organized into repeating units, called "costameres," that contain most of the membrane-bound spectrin (Repasky *et al.*, 1982; Craig and Pardo, 1983; Pardo *et al.*, 1983; Nelson and Lazarides, 1984; Porter *et al.*, 1992, 1997). Although other kinds of cells, such as fibroblasts, nerve, Schwann, and endothelial cells, are present in muscle tissue, the large mass and repetitive structural elements of myofibers have allowed us to determine some of the key features of their spectrin-based membrane skeleton. These features include the coassembly of α-fodrin, β-fodrin, and muscle β-spectrin into mixed complexes in developing muscle, the presence of only α-fodrin and muscle β-spectrin in adult myofibers, and the developmental regulation of expression of β-fodrin and β-spectrin that accounts in part for these changes.

Northern blot analyses indicate that as the expression of β-fodrin decreases during late embryonic and early postnatal development, expression of the muscle isoform of β-spectrin increases. Our analysis of the changes in β-spectrin was complicated by the presence of multiple transcripts of this protein. These were first described by Bloom *et al.* (1993), who reported sizes of 4.0, 7.2, 10.3, and 11.0 kb in adult mouse muscle. Our gels failed to resolve the 10.3- and 11-kb bands; the differences in size and number of the other transcripts are probably due to our use of different standards, as well as species and age differences. The transcript we observe at 3.5 kb is too small to encode full-length spectrin, and the protein(s) it encodes is still unknown. The 6.0-kb transcript may be large enough to encode a nearly full-length β-spectrin, perhaps related to the unusual β-spectrin that associates with acetylcholine receptors in cultured muscle cells (Bloch and Morrow, 1989; Daniels, 1990; Pumplin, 1995). The two transcripts we observed at 7.5 and 9.0 kb contain the erythrocyte 3′-alternative splice, recognized by a polymerase chain reaction product specific for this region. Even late in embryogenesis, when these transcripts are still present in muscle tissue, the erythrocyte isoform of β-spectrin is not expressed in myofibers, but is instead found in capillaries and surrounding tissue. The decrease in the erythroid alternatively spliced form (βIΣ1) around the time of birth in the rat is probably due to the steady decline in circulating reticulocytes that occurs during late prenatal and early postnatal life (Russell and Bernstein, 1968).

Coincident with the large increase in the 11.0-kb mRNA encoding the muscle isoform of β-spectrin, we observed a significant decrease in the mRNA encoding β-fodrin, consistent with the replacement of this subunit by muscle β-spectrin as skeletal muscle matures. Although α-fodrin might be expected to decrease together with β-fodrin, it in fact increases. It appears likely that the up-regulation of β-spectrin accounts for the increase of α-fodrin, as these two subunits associate with each other in skeletal muscle (Porter *et al.*, 1997).

Immunofluorescence provides complementary information on the spectrins in developing skeletal muscle. Indeed, its demonstration of a significant change in the organization of spectrin and fodrin at different times of development is striking. The relatively sparse and loosely organized membrane skeleton in E16 muscle suggests that this structure is just forming as myofibers develop from myotubes. This structure is also relatively undifferentiated in terms of its composition, as it contains both α- and β-fodrin subunits and β-spectrin in many of the same structures (e.g., Figure 6). Immunoprecipitations confirm that these three subunits associate in developing muscle (Figure 9). The presence of β-fodrin may facilitate the formation of premature membrane skeletal structures that could then serve as precursors of the more mature membrane skeleton that develops later. By contrast, adult muscle fibers contain an extensive, rectilinear array of β-spectrin at costameres that is more extensive than that of α-fodrin (e.g., Figure 7; Porter *et al.*, 1997). Thus, the fodrins appear to predominate in the irregular membrane skeleton of developing skeletal muscle fibers, while β-spectrin appears to predominate in the more extensively organized structures typical of the mature muscle.

Nelson and Lazarides (1983) previously reported that chicken muscle cells in tissue culture also undergo a change in the expression of the spectrins that is consistent, in some respects, with our observations. That study used cultured cells in which nonmuscle cells were relatively sparse, but it was complicated by the fact that the β-spectrin subunits were not fully characterized in birds. If the spectrins are handled in avian muscle as they are in rat muscle, then the subunits termed β', β, and γ by Nelson and Lazarides

should be equivalent to erythroid β-spectrin (βIΣ1), skeletal muscle β-spectrin (βIΣ2), and β-fodrin (βII), respectively. However, we were not able to detect erythroid β-spectrin (βIΣ1) in skeletal muscle fibers in any stage of muscle development.

Based on our results, we propose a developmental model for spectrin in skeletal muscle (Figure 10). At embryonic stages of muscle development when myoblasts and early myotubes predominate, only α- and β-fodrin are likely to be present in significant amounts at the plasmalemma. Weed (1996) has reported that C2C12 myoblasts express only these subunits, without β-spectrin. As differentiation proceeds (E15 to P7 in the rat), both fodrin subunits, as well as β-spectrin, are expressed and incorporated into membrane-associated structures, perhaps representing a transitional stage. Although the complex we have identified by immunoprecipitation at this stage is pictured as an heterotetramer in Figure 10, additional experiments will be needed to determine whether this is indeed the case, or whether other spectrin-associated proteins link heterodimers of α-fodrin and β-fodrin to heterodimers of α-fodrin and β-spectrin. If this in fact occurs, it must nevertheless be transient, because in adult muscle fibers all costameric structures contain β-spectrin, whereas only some contain α-fodrin (Figure 7; Porter *et al.*, 1997) and none contain β-fodrin. β-Fodrin is instead distributed through the remaining tissues of skeletal muscle, such as nerve and blood vessels, and so has no direct role in stabilizing the sarcolemma.

It seems likely that the switch from β-fodrin to muscle β-spectrin is part of the normal program of muscle differentiation. Skeletal fibers in the rat form in two stages: primary fibers between E15 and birth, and secondary fibers between E17 and P7 (Ross *et al.*, 1987). The number of skeletal muscle fibers therefore does not change greatly after birth, when the switch from β-fodrin to β-spectrin is completed. Early postnatal life is, however, the period when skeletal muscles become increasingly active and undergo a large increase in size (Harris, 1981). Our results suggest that the augmented expression of muscle β-spectrin and the decreased expression of β-fodrin, like the expression of many other genes in skeletal muscle, are linked to fiber activity and growth. It will be of interest to determine the nature of the *cis*-acting elements that control the genes encoding these two proteins and how they are influenced by innervation and contractile activity.

The significance of the increased presence of β-spectrin in adult muscle fibers is not yet clear. Perhaps β-spectrin is preferred in adult muscle because it may be capable of forming homo-oligomers (Woods and Lazarides, 1986) or because it can associate with the plasma membrane without always requiring a paired α subunit [e.g., Figure 7; see Porter *et al.* (1997), for a discussion]. This ability would permit skeletal muscle fibers to create membrane domains with different biochemical and biophysical properties, sequestering particular membrane proteins and providing distinct interactions with underlying structures that could stabilize the membrane during the contractile cycle. β-Fodrin may be less capable than β-spectrin of binding to the sarcolemma in the absence of α-fodrin and, if so, it would be less useful for these purposes. Experiments using cDNA transfection of normal and mutant skeletal muscle fibers, as well as studies of the ankyrins in skeletal muscle, may resolve these issues.

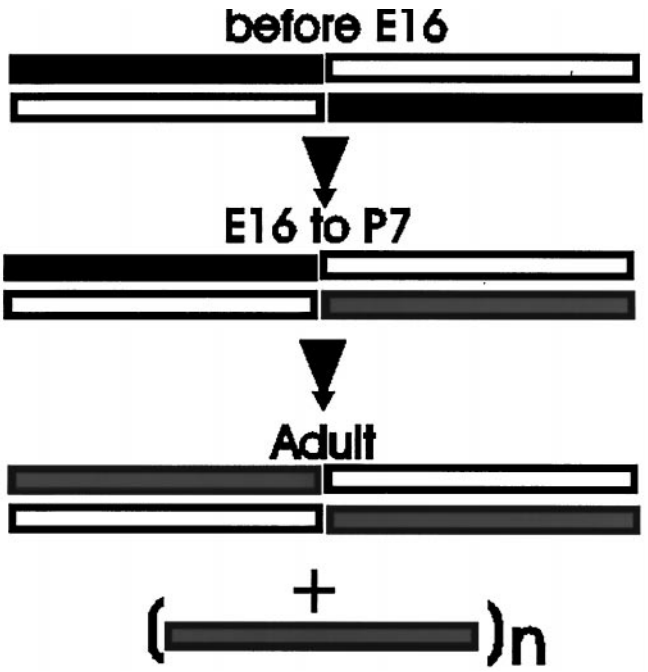

Figure 10. Proposed model of changes in spectrin during skeletal muscle development. Open bar, α-fodrin; solid bar, β-fodrin; shaded bar, muscle β-spectrin. Our results can most easily be explained by a progressive change in the composition of spectrin tetramers as muscle fibers develop. Specifically, we propose that embryonic myoblasts and early myotubes contain tetramers of α- and β-fodrin (Weed, 1996), but that mixed tetramers containing these two subunits together with muscle β-spectrin begin to form with the onset of myogenesis and persist through early postnatal life. Adult muscle fibers contain heteromers composed of only α-fodrin and β-spectrin as well as β-spectrin that assembles at the sarcolemma without α-fodrin (Porter *et al.*, 1997); the state of oligomerization of the latter population of β-spectrin is still unknown. This model does not include the proteins that bind to spectrin (e.g., actin, adducin, ankyrin, band 4.1) or other members of the spectrin superfamily that have not yet been identified, and so it may need to be revised when the associations of these proteins with spectrin are studied in mature and developing muscle fibers.

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