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A Nonerythroid Isoform of Protein 4.1R Interacts with Components of the Contractile Apparatus in Skeletal Myofibers

Aikaterini Kontrogianni-Konstantopoulos,* Shu-Ching Huang,*† and Edward J. Benz, Jr.*‡

*Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; and ‡Department of Molecular Biology and Genetics, The Johns Hopkins University, Baltimore, Maryland 21205

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The ~80-kDa erythroid 4.1R protein is a major component of the erythrocyte cytoskeleton, where it links transmembrane proteins to the underlying spectrin/actin complexes. A diverse collection of 4.1R isoforms has been described in nonerythroid cells, ranging from ~30 to ~210 kDa. In the current study, we identified the number and primary structure of 4.1R isoforms expressed in adult skeletal muscle and characterized the localization patterns of 4.1R message and protein. Skeletal muscle 4.1R appears to originate solely from the upstream translation initiation codon (AUG-1) residing in exon 2′. Combinations of alternatively spliced downstream exons generate an array of distinct 4.1R spliceoforms. Two major isoform classes of ~105/110 and ~135 kDa are present in muscle homogenates. 4.1R transcripts are distributed in highly ordered signal stripes, whereas 4.1R protein(s) decorate the sarcoplasm in transverse striations that are in register with A-bands. An ~105/110-kDa 4.1R isoform appears to occur in vivo in a supramolecular complex with major sarcomeric proteins, including myosin, α-actin, and α-tropomyosin. In vitro binding assays showed that 4.1R may interact directly with the aforementioned contractile proteins through its 10-kDa domain. All of these observations suggest a topological model whereby 4.1R may play a scaffolding role by anchoring the actomyosin myofilaments and possibly modulating their displacements during contraction/relaxation.

INTRODUCTION

Erythrocyte protein 4.1R is an ~80-kDa sulfhydryl-rich phosphoprotein that constitutes a major component of the red blood cell cytoskeleton (Ungewickell *et al.*, 1979). Protein 4.1R mediates the formation and maintenance of spectrin/actin complexes and contributes to anchoring the cytoskeleton to the overlying lipid bilayer (Bennett and Gilligan, 1993). Defects of 4.1R protein are accompanied by severe congenital hemolytic anemias, including hereditary elliptocytosis (Delaunay, 1995).

Four major domains of erythroid 4.1R have been identified after limited chymotryptic digestion (Leto and Marchesi, 1984): an NH_2-terminal ~30-kDa domain that mediates interactions with transmembrane proteins (Pasternack *et al.*, 1985; Marfatia *et al.*, 1995); a 16-kDa hydrophilic domain of unknown function; a highly charged 10-kDa domain that is involved in the formation of spectrin/actin complexes (Horne *et al.*, 1993; Schischmanoff *et al.*, 1995); and a 22/24-kDa acidic domain that interacts with several matrix proteins (Mattagajasingh *et al.*, 1999).

The prototypical ~80-kDa 4.1R protein, identified in mature erythrocytes, is only one of many isoforms that are generated by the single 4.1R gene via 1) complex alternative pre-mRNA splicing, 2) use of two distinct translation initiation codons, and 3) posttranslational modifications (Tang *et al.*, 1990; Conboy *et al.*, 1991; Subrahmanyam *et al.*, 1991; Huang *et al.*, 1993; Baklouti *et al.*, 1996). Use of an upstream initiation codon results in the generation of a 4.1R isoform that migrates with a molecular mass of ~135 kDa. This isoform carries an NH_2-terminal extension of 209 amino acids, called Headpiece (Hp), and has been detected during early erythropoiesis and in a number of nonerythroid tissues (Tang *et al.*, 1990). Moreover, a diverse collection of immunoreactive 4.1R polypeptides that range in size from 30 to 210 kDa have been identified by Western blotting in many types of nonerythroid cells (Anderson *et al.*, 1988).

Recently, three novel 4.1-like genes were added to the already complex NF2/ERM/4.1 gene family: 4.1G, 4.1N, and 4.1B (Peters *et al.*, 1998). 4.1G is widely expressed among

† Corresponding author. E-mail address: schuang@welch.jhu.edu.

different tissues and organs (Parra *et al.*, 1998), whereas 4.1N and 4.1B seem to be confined mainly in the peripheral and central neurons and the brain, respectively (Walensky *et al.*, 1999; Yamakawa *et al.*, 1999; Ye *et al.*, 1999; Parra *et al.*, 2000). A significant degree of homology within the 30-, 10-, and 22/24-kDa domains is shared by 4.1R and the three 4.1-like genes, whereas the NH_2 terminus and intervening sequences appear diverse and highly specific.

Although the major functions of the cytoskeletal ~80-kDa 4.1R protein have been characterized extensively in mature erythrocytes, little is known about the identity and potential roles of 4.1R isoforms in nucleated cells. Information about the intracellular distribution and protein partners of 4.1R in nonerythroid tissues has just started to emerge. 4.1R isoforms have been detected in both nuclear and cytoplasmic cellular compartments (Cohen *et al.*, 1982; De Carcer *et al.*, 1995; Krauss *et al.*, 1997) as well as in perinuclear regions, including centrosomal and Golgi structures (Leto *et al.*, 1986; Chasis *et al.*, 1993). Moreover, purified 4.1R interacts specifically in vitro with tubulin and skeletal muscle myosin (Correas and Avila, 1988; Pasternack and Racusen, 1989), whereas 4.1R polypeptides can associate functionally with components of the splicing apparatus (Lallena *et al.*, 1998) and the mitotic spindle pole during cell division (Mattagajasingh *et al.*, 1999). All of these observations indicate that distinct isoforms of 4.1R protein may exhibit specialized functions in different types of nonerythroid cells.

In the course of our efforts to identify the functional activities of nonerythroid 4.1R protein(s), we sought to characterize 4.1R in adult skeletal muscle. To this end, we identified the number and exonic composition of 4.1R isoforms present in skeletal myofibers and characterized the subcellular localization of 4.1R mRNA and protein(s). Furthermore, we demonstrated the presence of 4.1R in vivo in a supramolecular complex with major sarcomeric proteins, including myosin, α-actin, and α-tropomyosin, and showed that 4.1R can interact directly in vitro with the aforementioned contractile proteins through its 10-kDa domain.

MATERIALS AND METHODS

Animals and Tissue Preparation

Adult male and female 4.1R+/+ mice obtained from Drs. Mohandas and Conboy (Life Sciences Division, Lawrence Berkeley National Laboratory, University of California, Berkeley, CA) (Shi *et al.*, 1999) were anesthetized in a CO_2 chamber. Quadriceps muscle tissue was dissected directly and either frozen in liquid nitrogen for preparation of whole muscle homogenates or rinsed with 1× PBS for processing in immunolabeling experiments. To obtain whole protein lysates, frozen tissue was ground to powder in the presence of liquid N_2 and subsequently dissolved in triple lysis buffer (50 mM Tris, pH 7.5, 125 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% Na deoxycholate) in the presence of a protease inhibitor cocktail (Mattagajasingh *et al.*, 1999). Protein concentration was determined by a standard Bradford assay (Pierce, Rockford, IL). For immunolabeling experiments, longitudinally dissected muscle tissue was fixed in 10% formalin and embedded in paraffin blocks according to standard histology techniques. Semithin sections (3–4 μm) were prepared accordingly and used in in situ hybridization and immunofluorescence assays.

Generation of Antibodies

Four different polyclonal antibodies against distinct domains of 4.1R protein were used. Antiserum against synthetic peptides of segments of 16 kDa (TQAQTRQASALIDRPAFERC, nucleotides 1478–1539) and 22/24 kDa (GVLLTAQTITSETPSSTTTTKITKC, nucleotides 2123–2192) or to recombinant Hp extension were produced and affinity purified as described previously (Tang *et al.*, 1990; Baklouti *et al.*, 1997; Mattagajasingh *et al.*, 1999). Moreover, a peptide encoding exon 17a (VKKTSVLPSERKVGGPE, nucleotides 2358–2408) (Baklouti *et al.*, 1997) was used for generation of polyclonal antibodies as well (Zymed, South San Francisco, CA). The E17a antibodies were subsequently immunoaffinity purified with the use of the Sulfolink kit (Pierce).

RNA Preparation, cDNA Cloning, and DNA Dot Blot Analysis

Total RNA from adult mouse quadriceps muscle was obtained with the RNeasy Midi kit from Qiagen (Valencia, CA). Five micrograms of RNA were subjected to reverse transcription-PCR (RT-PCR) according to standard experimental protocols. The primers used in the PCR amplification were chosen to anneal to sequences present in exons 1 (primer a) and 21 (primer b) and are shown in Figure 1A (Table I). The PCR products were subcloned into TOPO TA vector (TOPO TA cloning kit, Invitrogen, Carlsbad, CA). Ninety-six bacterial colonies were randomly selected and grown in a 96-well microtiter plate to be analyzed in a DNA dot blot assay. 4.1R exon-specific oligonucleotide probes were digoxigenin-labeled according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN) and used to identify the presence of constitutive and alternatively spliced exons. Figure 1A (Table I) shows the sequences of the 4.1R exon-specific probes used. A number of clones (~15) were also analyzed by sequencing (T7 Sequenase Version 2.0 kit, Quick Denature plasmid, United States Biochemical, Cleveland, OH) with primers that spanned the entire length of the 4.1R cDNA.

In Situ Hybridization of Adult Skeletal Muscle Sections

In situ hybridization of mouse quadriceps muscle tissue was performed with the use of digoxigenin-labeled oligonucleotide probes (Nonradioactive In Situ Hybridization Application manual, Boehringer Mannheim) that corresponded to sequences encoded either by exon $17a_{(2358–2408)}$ or by a segment of exon $18_{(2441–2465)}$ in the antisense orientation (Baklouti *et al.*, 1997). Muscle sections were deparaffinized and rehydrated according to standard histology techniques. The in situ hybridization procedure was performed essentially as described in the Nonradioactive In Situ Hybridization Application manual with some modification, including heat treatment of sections in a microwave oven in the presence of 10 mM diethyl pyrocarbonate-sodium citrate, pH 6.0, for 15 min (Hougaard *et al.*, 1997). Color reaction was performed at 25°C in the dark for 2–3 h. The developed slides were mounted with Crystal Mount (Biomeda, Foster City, CA) and processed for microscopic examination and photographic documentation under a Nikon (Garden City, NY) microphot FXA phase-contrast microscope. In control experiments, either the aforementioned oligonucleotide probes were used in the sense orientation or they were completely omitted.

Western Blot Analysis

Skeletal muscle lysates (50–100 μg) were analyzed by 8% SDS-PAGE and subsequently electrotransferred onto a polyvinylidene difluoride membrane (New England Nuclear Life Science Products, Boston, MA). As primary antibodies, affinity-purified anti-4.1R E17a, 22/24 kDa, and Hp immunoglobulin Gs (IgGs) were used at a 1:250 dilution. The immunoreactive bands were visualized with the use of the ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

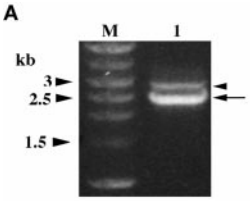

Table I

pra(nts1447-1464)-S : 5'GACAACGACTACTTCTGG *1
prb(nts 4070-4084)-AS: 5'CCAGACATGTCAGTGACCAA *1
mEx2'(nts1465-1481): 5'GAACATCATGACAACAG *1
mEx2(nts1547-1570): 5'GCCACAAACTCAGGCCAACAAGAA *1
mEx4(nts2221-2246): 5'GAATGTGTTGTAGAGAAACATGCTA *1
mEx8(nts2637-2662): 5'AGGAACTTGAAGAAAAGGTCATGGAA *1
mEx13(nts3361-3379): 5'GCCCACAGAAGCATGGAAG *1
mEx16(nts3482-3510): 5'AAGAGAGAGAGACTAGATGGTGAAAACAT *1
hEx17a(nts2367-2395): 5' ACTTCTGTCCTACCCTCGGAAAGAAAGGT *2
hEx17b(nts257-282):5'TAGGAGTGGAGGAACAGGCCAGTGCC *3
mEx18(nts:3751-3775):5'CTCAGATACTGCCAATGCTGTGAAA *1
mEx19(nts3930-3949):5'CCACGACACAGATTACCAAG *1
mEx20(nts3958-3975):5'AGGTGGGATTTCTGAGAC *1

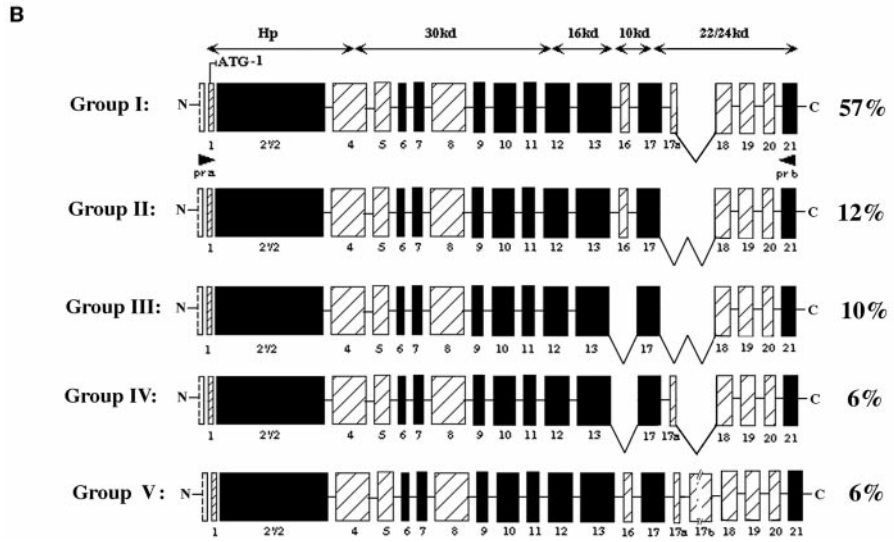

Figure 1. Characterization of 4.1R messages in adult mouse skeletal muscle by RT-PCR analysis. (A) One-percent agarose gel electrophoresis revealed the presence of two amplification bands of ~2.6 and ~3 kb (arrow and arrowhead, respectively; for detailed description, see RESULTS). The table shows the nucleotide sequences of primers a and b, used in the PCR amplification assay, and the exon-specific oligonucleotide probes, used in the screening process of the skeletal muscle 4.1R cDNA library. h, human; m, mouse; nts, nucleotides; *1, GenBank accession number L00919; *2, Baklouti *et al.*, 1997; *3, Schischmanoff *et al.*, 1997. (B) Diagram and percentile prevalence of the major 4.1R isoforms identified in adult mouse skeletal myofibers. Previously characterized constitutive sequences are indicated as black boxes, and alternatively spliced cassettes are depicted as shaded boxes.

Immunofluorescence Combined with Confocal Microscopy

Mouse quadriceps muscle sections were prepared as described above (see Animals and Tissue Preparation) and processed for deparaffinization and rehydration. To unmask any hidden epitopes, the samples were microwaved for 15 min in the presence of 10 mM citrate buffer, pH 6.0. After blocking in PBST (1× PBS, 0.2% Tween 20) plus 3 mg/ml BSA for 2 h at 25°C, the sections were incubated in the same solution, in the presence of the appropriate primary antibodies for 12 h, at 4°C in a humid chamber. In single-label experiments, the following 4.1R antibodies were used: anti-Hp (1:10), anti-E17a (1:50), anti-22/24 kDa (1:50), and anti-16 kDa (1:50); in double-label experiments, anti-myosin MF20 IgG (1:10) (Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA), anti-actin JLA20 IgM (1:500) (DSHB), and anti-α-actinin IgG (1:500) (Sigma Chemical, St. Louis, MO) were also added along with the anti-E17a antibody. After extensive washing in PBST, the sections were counterstained for 1 h at 37°C with FITC-conjugated anti-rabbit IgG (Zymed), rhodamine-conjugated anti-mouse IgG (Pierce), or rhodamine-conjugated anti-mouse IgMμ (Pierce). After several washes in PBST, slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and processed for microscopic observation under either a Nikon microphot FXA microscope or a laser scanning confocal system (Noran Instruments, Middleton, WI) combined with a Zeiss (Thornwood, NY) Axiophot microscope through a 100× oil-immersion objective. In a series of control immunodepletion experiments, anti-E17a, anti-22/24 kDa, and anti-16 kDa antibodies were preabsorbed with 100 μg of their respective antigens per milliliter of diluted serum for 8–12 h at 4°C with gentle rocking and subsequently applied to muscle sections.

Coimmunoprecipitation and Immunoblotting

Six to eight μg of anti-4.1R E17a and 22/24 kDa as well as 2 μl of anti-myosin-M7523 (Sigma) IgGs were allowed to interact with protein A-Sepharose-6MB beads (150 μl of a 50% suspension; Amersham Pharmacia Biotech) in the presence of 1× PBS at 4°C with gentle rocking for 12 h. Additionally, 6–8 μg of anti-tropomyosin-CH1 IgG1 (DSHB) were incubated under the same conditions with protein G-Sepharose-4/Fast Flow (Pierce). Total skeletal muscle homogenates (500 μg) were precleared with 150 μl of the appropriate type of agarose beads in triple lysis buffer, in the presence of a protease inhibitor cocktail, on a 4°C rocker for 2 h. Subsequently, the antibodies bound to the beads were incubated with the precleared muscle lysate by gentle rocking at 4°C for 4 h. At the end of the incubation period, the samples were centrifuged for 15–20 s at

14,000 × *g* at 4°C. The supernatants were collected and stored at −20°C, whereas the beads were washed extensively with several changes of triple lysis buffer by rocking at 4°C. At the end of the washings, the proteins were solubilized in 80 μl of 2× Laemmli sample buffer and boiled for 5 min. The immunoprecipitates were analyzed on 8% SDS-PAGE and processed for immunoblotting with the indicated antibodies (see RESULTS). In some cases, the gels were stained with GelCode SilverSNAP (Pierce) for direct visualization of the immunoprecipitates. Protein bands of interest were quantified by NIH Image software. In parallel control experiments, the antibodies were either omitted or replaced by rabbit or mouse IgGs to determine nonspecific interactions.

Overlay Assay

GST fusion peptides of the different 4.1R domains were prepared as described previously (Mattagajasingh *et al.*, 1999). Equivalent amounts of GST-4.1R fusion proteins as well as GST protein alone were separated on 10% SDS-PAGE and either stained with Coomassie blue dye or electrotransferred onto a polyvinylidene difluoride membrane. Alternatively, 50 μg of adult skeletal muscle homogenates was subjected to 8% SDS-PAGE followed by electroblotting. Subsequently, the membranes were incubated in blocking buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 0.1% Tween 20, 3% BSA, 0.5% gelatin, 2 mM DTT, 0.5% NP-40) for 12 h at 4°C. Four GST-4.1R replica blots were overlaid with 3 μg/ml native heavy meromyosin (HMM) (Cytoskeleton, Denver, CO), sarcomeric F-actin (Cytoskeleton), skeletal muscle tropomyosin (Sigma), or sarcomeric α-actinin (Cytoskeleton), whereas three identical skeletal muscle lysate blots were overlaid with 3 μg/ml GST-4.1R-10 kDa polypeptide plus or minus exon 16 or GST protein alone in binding buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 2 mM ATP, 1% BSA, 0.25% gelatin, 2 mM DTT, 0.5% NP-40) for 3–5 h on a 4°C rocker. At the end of the incubation period, the membranes were washed four to six times for 20 min per wash with washing buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 2 mM DTT, 1% NP-40) at 4°C and once with TBST (15 min) to equilibrate to the immunodetection system. Subsequently, the membranes were processed for immunoblotting with the use of the appropriate antibodies, as indicated in RESULTS.

RESULTS

Structural Analysis of the Skeletal Muscle 4.1R Transcripts by RT-PCR

To characterize the 4.1R mRNA isoforms present in adult skeletal muscle, total RNA from mouse origin was subjected to RT-PCR analysis (Figure 1A). The primers set were chosen to amplify full-length coding sequences originating from either the upstream translation initiation codon (AUG-1), present in exon 2′, or the downstream AUG-2, residing in exon 4. Thus, the sense primer (primer a) annealed at the end region of exon 1, whereas the antisense primer (primer b) was complementary to sequences present at the end of exon 21 (Figure 1A shows the nucleotide sequences of primers a and b). A major amplification product was obtained that exhibited the expected size of ~2.6 kilobases (kb), whereas a less prominent band of ~3 kb was also detected (Figure 1A, lane 1, arrow and arrowhead, respectively).

To identify whether the obtained amplification bands corresponded to single mRNA species or a diverse population of 4.1R transcripts with similar sizes, the PCR products were subcloned and two approaches were used to characterize individual subclones. First, a number of representative clones (~15) were completely sequenced using primers that spanned the entire length of 4.1R cDNA; second, a mini 4.1R cDNA library was constructed. Consequently, DNA dot blot analysis of 96 randomly selected independent clones was performed with the use of digoxigenin-labeled, exon-specific oligonucleotide probes (Figure 1A and Table I show the sequences of the exon-specific primers used).

The 4.1R cDNA library was initially screened with probes specific for the constitutive exons 2 and 13 to ascertain the successful design and progression of the subcloning experiment. Subsequently, exon-specific probes for alternatively spliced sequences were used. Notably, all of the 96 4.1R cDNA clones (100%) included exon 2′, indicating that in skeletal muscle 4.1R polypeptides originate predominantly, if not exclusively, from AUG-1. Five major 4.1R isoforms were identified (Figure 1B). The most common one constituted 57% of the total 4.1R cDNAs analyzed and included all of the previously characterized constitutive exons (Baklouti *et al.*, 1997) and the alternatively spliced cassettes 4, 5, 8, 16, 17a, 18, 19, and 20 (Figure 1B, group I). The second major group (12%) encoded isoforms that skipped only exon 17a (Figure 1B, group II), whereas 10% excluded both exons 16 and 17a (Figure 1B, group III). Moreover, 6% of the whole cDNA population was missing solely exon 16 (Figure 1B, group IV), whereas another 6% included exon 17b, which has been shown to be highly expressed in cells of epithelial origin (Figure 1B, group V) (Schischmannoff *et al.*, 1997). The inclusion of exon 17b (450 nucleotides) accounts for the presence of the larger PCR product (Figure 1A, arrowhead), as also confirmed by sequence analysis.

We also detected additional minor 4.1R mRNA species with a different structural organization. These constituted 9% of the entire 4.1R cDNA population. Specifically, 3% skipped exon 17a but included exon 17b, 1% excluded solely exon 5, and another 1% skipped both exons 5 and 17a. Exon 8 was absent in 1% of 4.1R messages, whereas an additional 1% was missing both exons 8 and 17a. 4.1R isoforms that excluded either exon 19 (1%) or both exons 17a and 19 (1%) were identified as well. Finally, previous studies from our laboratory along with our sequencing data indicated that mRNAs including the alternatively spliced cassettes encoded by exons 14 and 15 are not, for the most part, present in adult skeletal muscle (Baklouti *et al.*, 1997).

4.1R mRNA Exhibits a Highly Ordered Distribution Pattern in Adult Skeletal Muscle

To investigate the localization pattern of 4.1R messages in adult myofibers, in situ hybridization was used (Figure 2). The sequences encoded by exon 17a or a segment of exon 18 (see MATERIALS AND METHODS) in the antisense orientation served as probes. In longitudinal sections of skeletal myofibers, 4.1R messages appeared as highly ordered signal stripes (Figure 2, A and B, arrows). Furthermore, when the distribution of this mRNA species was examined in a series of cross-sections, a definite, punctate staining within the sarcoplasm was observed (Figure 2C, arrows). An occasional staining around the periphery of myofibers was also detected in both longitudinal sections and cross-sections (Figure 2, A and C, arrowheads). However, the great majority of the signal obtained was consistently detected within the sarcoplasm. When exon 17a was used in the sense orientation, no specific signal was obtained (Figure 2D).

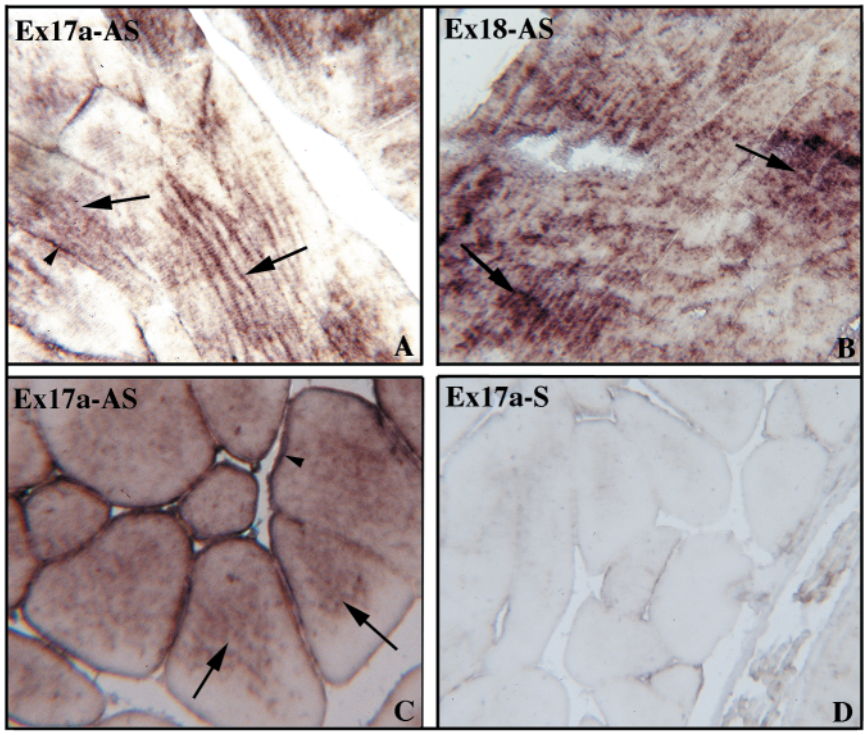

Figure 2. In situ hybridization photomicrographs illustrating the distribution pattern of 4.1R transcripts in longitudinal sections (A, B, and D) and cross-sections (C) of skeletal muscle. Two different 4.1R oligonucleotide probes were used in the antisense orientation: E17a (A and C) and E18 (B). 4.1R mRNA is localized predominantly within the sarcoplasm (A–C, arrows), whereas an occasional staining at the periphery of skeletal fibers was also detected (A and C, arrowheads). The specific signal was eliminated completely when E17a probe was used in the sense orientation (D). Magnification, ×40.

Two Major 4.1R Isoforms Are Present in Adult Skeletal Muscle

A series of immunoblot assays was performed to identify the 4.1R isoforms expressed in skeletal muscle, with the use of a panel of 4.1R-specific antibodies. These included anti-E17a, anti-22/24 kDa, and anti-Hp (Figure 3A). A predominant ~105/110-kDa polypeptide was recognized by anti-E17a and anti-22/24 kDa antibodies (Figure 3A, double arrowhead). An additional ~135-kDa immunoreactive band was also observed by both antibodies, although at lower expression levels (Figure 3A, arrow), whereas anti-22/24 kDa detected an ~160-kDa protein as well (Figure 3A, arrowhead). This ~160-kDa 4.1R isoform is currently being characterized by our laboratory and has been described in a number of cell lines and adult mouse tissues (S.C. Huang, J. Hartenstein, and E.J. Benz, Jr., unpublished data). Nevertheless, when anti-Hp antibody was used, a prevalent band of ~135 kDa was identified and, at lower levels, an ~105/110-kDa isoform (Figure 3A, arrow and double arrowhead, respectively). An ~86-kDa polypeptide was also detected (Figure 3A, open arrowhead) that may be another alternatively spliced product of the 4.1R gene.

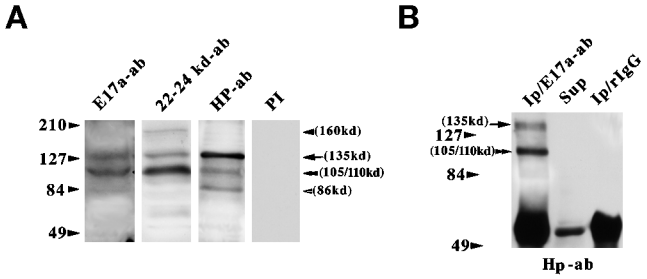

Figure 3. Characterization of 4.1R isoforms present in skeletal muscle homogenates by Western blotting (A) and immunoprecipitation (B). Three different 4.1R antibodies were used: anti-E17a, anti-22/24 kDa, and anti-Hp; preimmune serum (PI) served as a negative control. In B, the immunoprecipitation assay was performed in the presence of either anti-E17a antibody or control rabbit IgG, as indicated. The blot was subsequently probed with anti-Hp antibody. Sup, supernatant fraction of anti-E17a immunoprecipitate. One-half of the immunoprecipitates (Ip) and one-eighth of the supernatant fraction were loaded onto the respective lanes. The arrows denote the migration position of the ~135-kDa isoform, and the double arrowheads point to the ~105/110-kDa protein. The closed and open arrowheads indicate the ~160- and ~86-kDa polypeptides, respectively.

Together, these findings suggested that the three different 4.1R antibodies recognize the two major isoforms (~135 and ~105/110 kDa) with distinct specificities. This observation prompted us to verify further the identity of the observed immunoreactive bands. Thus, we performed an immunoprecipitation assay with the use of total muscle lysates and E17a antibody or control rabbit IgGs (Figure 3B). An aliquot of the immunoprecipitates was subsequently probed with anti-Hp antibody. Both the ~105/110- and ~135-kDa isoforms were efficiently immunoprecipitated by anti-E17a and specifically recognized by anti-Hp antibody (Figure 3B, double arrowhead and arrow, respectively). No immunoreactive bands were detected in the control rabbit IgG immunoprecipitate.

Protein 4.1R Exhibits a Periodic Pattern of Cross-Striations within the Myoplasm

We used immunofluorescence microscopy to study the intracellular distribution of 4.1R in longitudinal sections of

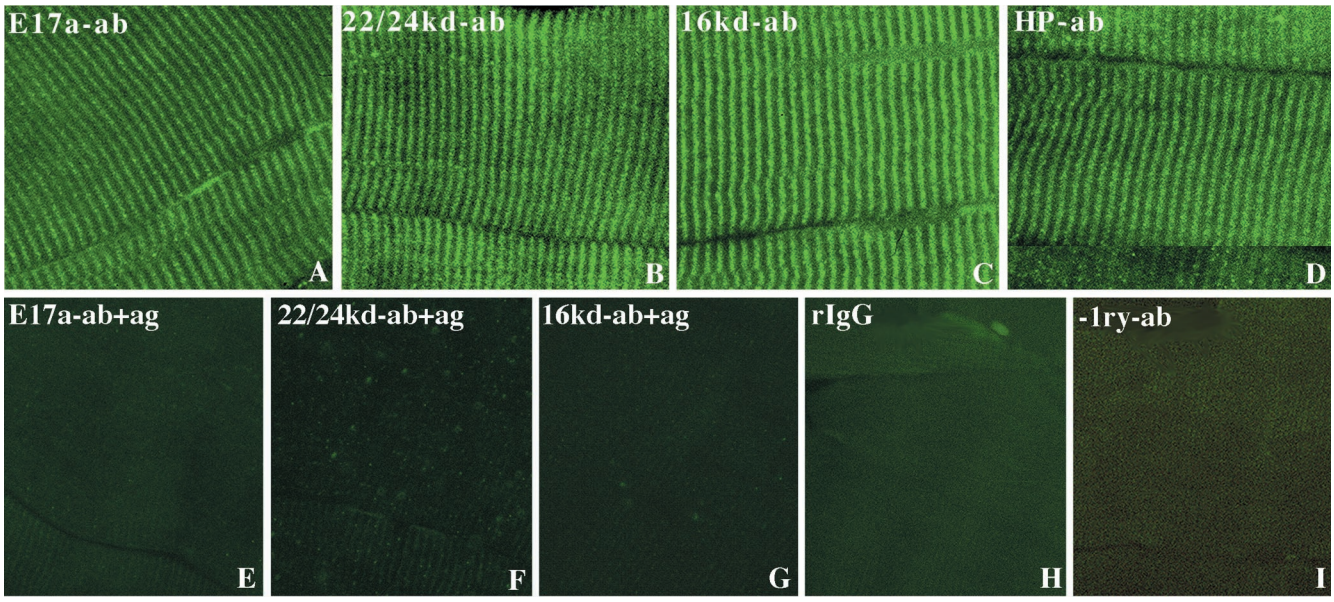

Figure 4. Analysis of the localization pattern of 4.1R protein isoform(s) in adult skeletal muscle by immunofluorescence microscopy. A panel of four 4.1R antibodies was used with distinct domain specificities: anti-E17a (A), anti-22/24 kDa (B), anti-16 kDa (C), and anti-Hp (D). A highly ordered pattern of cross-striations was observed in all cases. In control immunodepletion experiments, anti-E17a, anti-22/24 kDa, and anti-16 kDa antibodies were preabsorbed in the presence of their respective antigens (panels E, F, and G, respectively). Furthermore, when the primary antibodies were either replaced by rabbit IgGs (H) or omitted (I), no specific signal was detected. Magnification, ×40.

adult skeletal myofibers. Four different 4.1R antibodies were used; each decorated transverse striations in a punctate pattern (Figure 4, A–D). Preabsorption of the primary antibodies with their respective antigens abolished staining completely (Figure 4, E–G), whereas no signal was detected when primary antibodies were either replaced by rabbit IgGs or omitted (Figure 4, H and I, respectively).

To align the transverse periodic staining of 4.1R relative to major components of the sarcomere, we performed a series of double immunolabeling experiments analyzed by three-dimensional confocal microscopy. Anti-4.1R E17a along with anti-myosin (A-band marker) or anti-α-actinin (Z-disk marker) antibodies were used (Figure 5). Approximately 50 sections of 0.25 μm each were obtained. The stack of sections was digitally assembled (Figure 5, A–C, D, and E), and a transverse view of the immunostained tissue was taken along the xx′ axis (Figure 5, A′–C′ and D′–F; the plane of the transverse view is indicated by the red line marked xx′). Myosin staining was visualized as thick striations of ~1.5 μm occupying the A-bands (Figure 5B), whereas the Z-disk marker α-actinin showed a periodic pattern of thin striations crossing the myoplasm at ~2-μm intervals (Figure 5E). Superimposed images of 4.1R and myosin revealed complete overlapping (Figure 5, A–C and A′–C′; overlapping areas appear yellow). Similar analysis of 4.1R and α-actinin exhibited distinct distributions for the two proteins, with no apparent regions of costaining (Figure 5, D, E, and D′–F). Together, these findings indicate that protein 4.1R is in register with the thick myosin filaments occupying the A-bands. A schematic presentation of the apparent distribution of 4.1R along the A-band is shown in Figure 5G, where 4.1R molecules are depicted as green particles.

Protein 4.1R Associates with Major Components of the Sarcomere In Vivo

The presence of protein 4.1R along the A-bands prompted us to investigate whether native 4.1R can associate in vivo with major contractile components. For this reason, we performed a series of coimmunoprecipitation (coIp) assays that used total muscle homogenates and anti-E17a or anti-22/24 kDa antibodies. Both 4.1R antibodies immunoprecipitated specifically and efficiently the ~105/110-kDa 4.1R isoform (Figure 6, A and E, respectively, arrows), whereas control rabbit IgGs did not precipitate any immunoreactive band. The longer exposure time of the two immunoblots shown in A and E revealed the presence of the ~135-kDa polypeptide as well (our unpublished observations). The A-bands are composed mainly of myosin filaments in addition to portions of overlapping actin filaments, which reside at the cross-bridges-bearing zone (C-zone) (Figure 5G). Consequently, we analyzed the anti-E17a and anti-22/24 kDa immunoprecipitates for the presence of the major sarcomeric proteins myosin (210 kDa, MF20-ab) and α-actin (43 kDa, JLA20-ab) and the actin-associated protein tropomyosin (36/39 kDa, CH1-ab). All three contractile proteins were detected readily (Figure 6, B and F, C and G, D and H, respectively, arrows). In the case of tropomyosin, the α isoform (36 kDa) was mainly observed, whereas only a minor fraction of the β isoform (39 kDa) was identified. Thus, 4.1R appears to possess different affinities for the α and β polypeptides, interacting mainly with α. In control coIp experiments in which rabbit IgGs were used, neither myosin, α-actin, nor α-tropomyosin was precipitated (Figure 6, B and F, C and G, D and H, respectively).

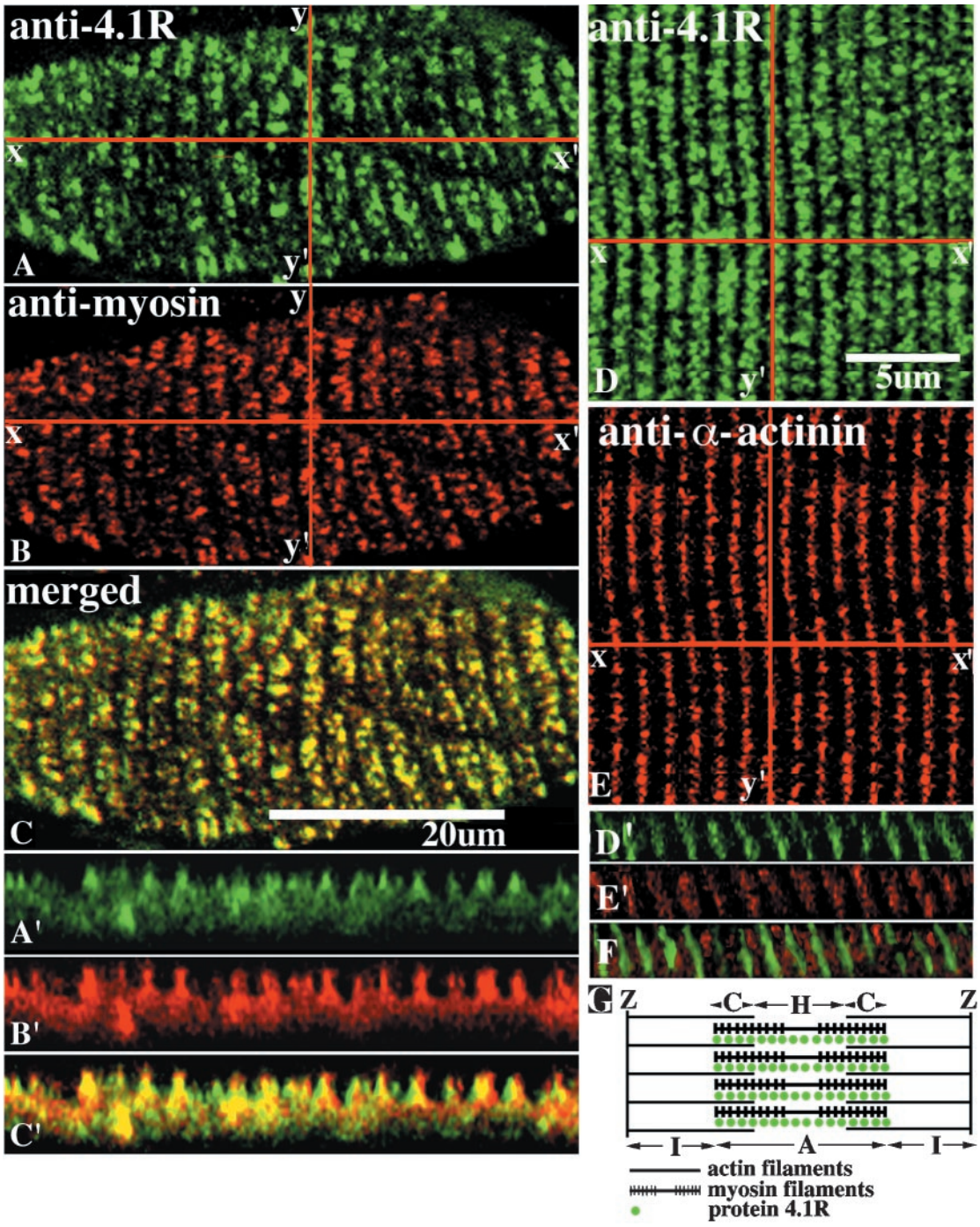

Figure 5. Characterization of 4.1R distribution relative to markers for the A-band and Z-disk by three-dimensional confocal microscopy. Coincident staining between myosin and 4.1R indicated that the latter is in register with A-bands (A–C and A′–C′). No apparent areas of colabeling between 4.1R and α-actinin (Z-disk marker; D, E, and D′–F) were observed. (G) Schematic presentation of the presence of 4.1R along the A-band; for simplicity of presentation, only the myosin and actin filaments are shown. Z, Z-discs, defining the limits of a sarcomere; I, I-band, consisting of actin filaments and associated proteins; A, A-band, composed mainly of myosin along with portions of overlapping actin filaments present at C-zones; H, H-zone, residing in the center of A-band. Magnification, ×100.

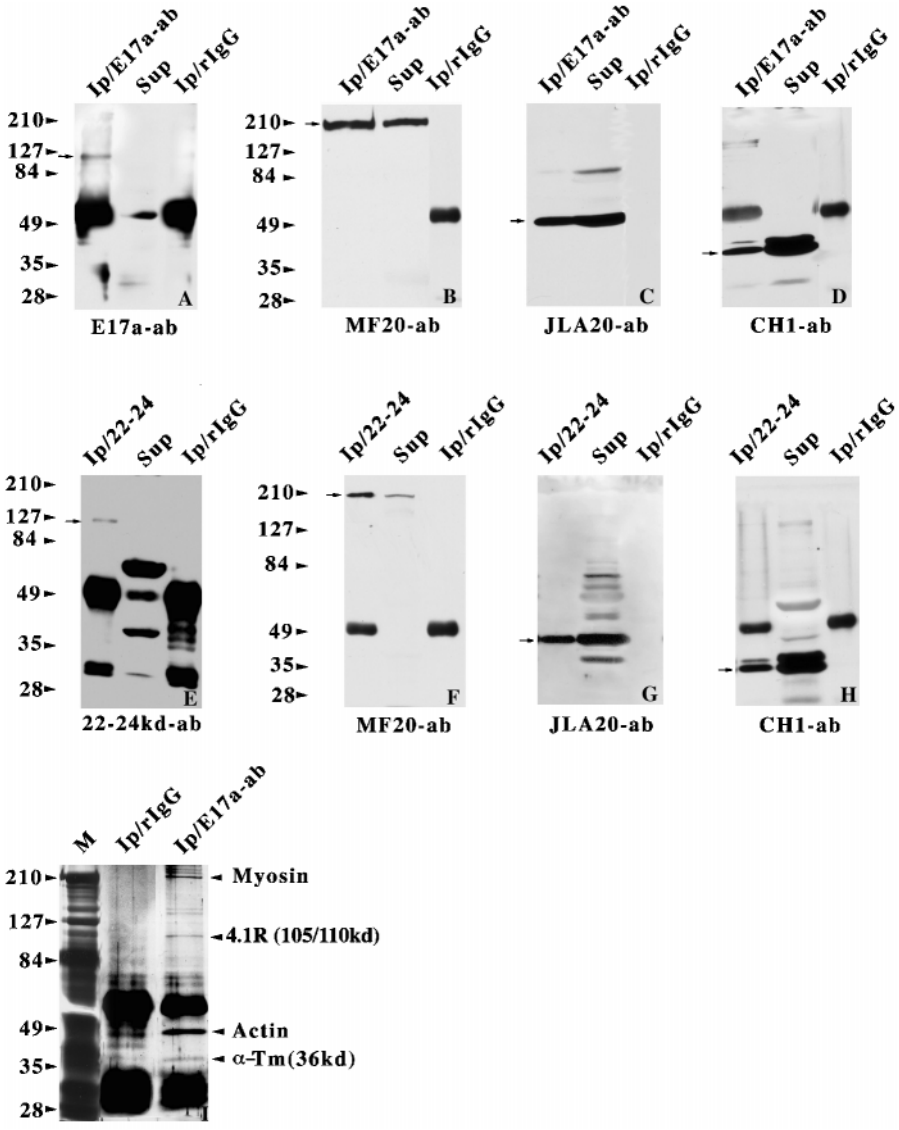

Figure 6. Coimmunoprecipitation of protein 4.1R and myosin, α-actin, and tropomyosin from adult skeletal muscle homogenates. The 4.1R anti-E17a antibody (A, B, C, D, and I) and anti-22/24 kDa antibody (E, F, G, and H) along with rabbit IgG were used in the immunoprecipitation assays. The immunoprecipitates were analyzed by immunoblotting with anti-E17a (A), anti-22/24 kDa (E), anti-myosin MF20 mAb (B and F), anti-actin JLA20 mAb (C and G), and anti-tropomyosin CH1 mAb (D and H). The arrows denote the migration positions of the ~105/110-kDa 4.1R protein (A and E), myosin (B and F), α-actin (C and G), and tropomyosin (D and H). One-fourth of the immunoprecipitates (Ip) and one-eighth of the supernatant fractions (Sup) were loaded onto the lanes as indicated. (I) An aliquot of either rabbit IgG or anti-E17a immunoprecipitates was subjected to silver staining. M, silver-stained molecular mass protein standards. The arrowheads point to the positions of protein 4.1R, myosin, α-actin, and α-tropomyosin.

To estimate the molar ratio between 4.1R and myosin, α-actin, and α-tropomyosin, an aliquot of the anti-E17a immunoprecipitate was analyzed by SDS-PAGE followed by silver staining. This approach appeared to us more reliable than analysis of the respective immunoblots because of the differential affinity that the antibodies can exhibit against their antigens. Approximately 10 protein bands were detected with distinct molecular masses and different signal intensities (Figure 6I). The bands that correspond to the ~105/110-kDa 4.1R isoform, as well as to myosin, α-actin, and α-tropomyosin, were readily identified and are shown in Figure 6I. A picture of the silver-stained gel was subsequently scanned, and the protein bands of interest were quantified by NIH Image software. The molar ratio between 4.1R and myosin, α-actin, and α-tropomyosin represents average values from three anti-E17a coIp experiments and was found to be 0.8:0.85:5:2.25. The values obtained from the three coIp assays exhibited a difference of 2–5%. Similar estimations about the relative amounts of the aforementioned proteins were obtained when the anti-22/24 kDa immunoprecipitates were analyzed accordingly (our unpublished observations).

The results obtained so far suggest that a 105/110-kDa 4.1R isoform occurs in vivo in a supramolecular complex with major constituents of the contractile apparatus. To further confirm this finding, a series of reverse coIp experiments were performed. The respective immunoprecipitates were analyzed for the presence of 4.1R protein with the use of the anti-Hp antibody. As has been described (Figure 3), this 4.1R antibody recognizes the ~135-kDa isoform with higher affinity compared with the ~105-kDa protein. Thus, if the ~135-kDa isoform was present in the myosin, α-actin, or tropomyosin immunoprecipitates, it would be detected efficiently.

When a polyclonal antibody against myosin and a mAb against tropomyosin were used, a major fraction of the respective proteins was precipitated efficiently (Figure 7, A and B,

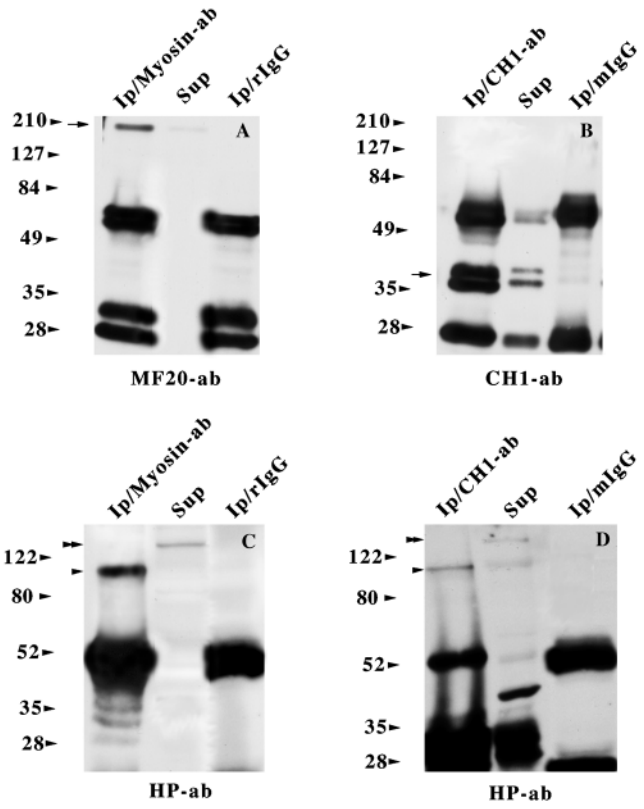

Figure 7. Reverse coimmunoprecipitation assays of sarcomeric myosin as well as tropomyosin and protein 4.1R from total skeletal muscle lysates. An anti-myosin polyclonal antibody (A and C) and anti-tropomyosin CH1 mAb (B and D) were used in the immunoprecipitation assays, along with control rabbit (A and C) and mouse (B and D) IgGs. The immunoprecipitates were analyzed by Western blotting with anti-myosin MF20 mAb (A), anti-tropomyosin CH1 mAb (B), and anti-4.1R Hp antibody (C and D). One-sixth of the immunoprecipitates (Ip) were loaded in A and B, and one-half of the same immunoprecipitates were loaded in D. In all cases, one-eighth of the supernatant fractions (Sup) were loaded. The presence of myosin (A) and tropomyosin (B) is indicated by arrows, whereas the ~105/110- and ~135-kDa 4.1R isoforms are denoted by single and double arrowheads, respectively (C and D).

respectively, arrows). Subsequent immunoblotting of these immunoprecipitates with anti-4.1R Hp antibody revealed the presence of a main band of ~105/110 kDa (Figure 7, C and D, arrowheads), whereas in the supernatant fractions both the ~135-kDa and the ~105/110-kDa isoforms were detected (Figure 7, C and D, double and single arrowheads, respectively). When we immunostained replicas of these blots with anti-22/24 kDa antibody, we obtained identical results (our unpublished observations). Furthermore, no immunoreactive bands were observed when either rabbit or mouse IgGs were used in control experiments (Figure 7, A and C, B and D, respectively). Several antibodies were used against sarcomeric α-actin. However, the available anti-α-actin antibodies belong to the IgM class, which shows very limited binding efficiency to any of the commercially available substrates (protein A, protein G, protein L, or Mannan binding protein (MBP) column). Nevertheless, all of these observations support the notion that a 4.1R isoform, presumably the ~105/110-kDa protein, associates in vivo with sarcomeric myosin, α-actin, and α-tropomyosin in a highly specific protein complex.

The 10-kDa Domain of Protein 4.1R Is Responsible for the Interaction with Myosin, α-Actin, and Tropomyosin

To investigate whether protein 4.1R interacts directly with myosin, α-actin, and tropomyosin and to identify the domain that is responsible for each individual association, a blot overlay assay was used. For this reason, we generated GST fusion proteins of the 4.1R domains, including Hp, 30 kDa, 16 kDa, 10 kDa, and 22/24 kDa plus or minus exon 17a. The authenticity of these fusion polypeptides was verified in a series of immunoblot assays with the appropriate antibodies (our unpublished observations). Purified skeletal muscle HMM, α-actin, and tropomyosin were used (Figure 8A). Equivalent amounts of GST-4.1R fusion proteins as well as GST protein alone were analyzed by SDS-PAGE (Figure 8B) and overlaid with native filamentous α-actin (F-actin), HMM, and tropomyosin. The binding of each sarcomeric protein was analyzed by Western blotting with the use of the corresponding antibodies (F-actin/JLA20 mAb, HMM/MF20 mAb, and tropomyosin/CH1 mAb; Figure 8, C, D, and E, respectively). All three sarcomeric proteins interacted directly with 4.1R. Interestingly, a specific association with the 10-kDa domain of 4.1R was revealed for all three proteins. To verify this observation and rule out the possibility of nonspecific binding, we performed a control overlay assay. A replica GST-4.1R blot was overlaid with α-actinin followed by immunodetection with an anti-α-actinin mAb (Figure 8F). No specific interaction between α-actinin and any of the 4.1R domains was identified.

Because the 10-kDa domain is responsible for the association of 4.1R with the sarcomeric proteins myosin, α-actin, and tropomyosin, we next attempted to identify which exon(s) are involved in each individual interaction. The 10-kDa domain consists of the previously identified alternatively spliced exon 16 and the majority of the constitutive exon 17 (Baklouti *et al.*, 1997; RT-PCR data herein). Thus, a GST-10 kDa fusion protein that is missing exon 16 was also constructed. Overlay assays were then performed with GST-10 kDa fusion peptides that either carry or miss exon 16 as well as GST protein alone (Figure 9A). The absence of exon 16 does not affect the interaction between F-actin and the 10-kDa domain (Figure 9B), because a strong and specific signal was obtained in both the presence and the absence of this nucleotide cassette. Surprisingly, though, the absence of exon 16 significantly augmented the binding affinity of HMM to the 10-kDa domain (Figure 9C). Finally, a rather moderate but consistent decrease of ~15%, as estimated by NIH Image software, was observed in the interaction of tropomyosin with the 10-kDa domain when exon 16 was absent (Figure 9D). This finding implies that some of the residues encoded by exon 16 might be involved in the association between 4.1R-10 kDa and tropomyosin along with sequences carried by exon 17.

To further verify the results obtained from the series of overlay experiments (Figures 8 and 9), we challenged our experimental protocol by actually performing the reverse assay (Figure 10). Thus, three identical blots of skeletal mus-

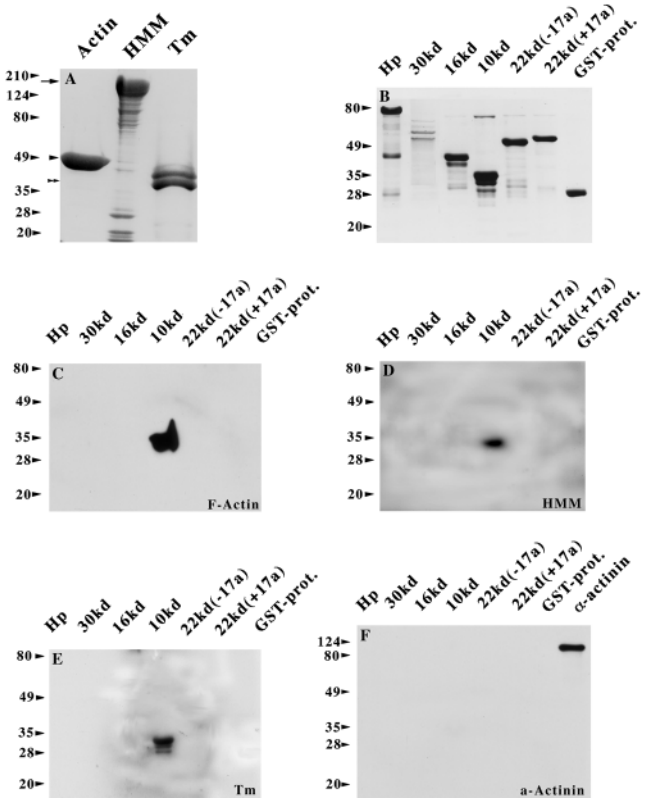

Figure 8. Blot overlay assays of the 4.1R domains and the sarcomeric proteins F-actin, HMM, and tropomyosin (Tm). (A) Aliquots of 10 μg of purified skeletal muscle F-actin, HMM, and tropomyosin are shown by Coomassie blue staining. (B) Equivalent amounts of GST-4.1R fusion peptides and GST protein alone were separated by SDS-PAGE and stained with Coomassie blue as well. The GST-Hp fusion protein migrates as an ~80-kDa protein instead of ~55 kDa, which is presumably the result of posttranslational modifications that take place in the bacterial expression system. Furthermore, the 30-kDa domain is highly insoluble; thus, only limited amounts could be obtained. Some smaller bands that are detected in GST-Hp, GST-16 kDa, and GST-10 kDa are presumably the results of degradation. Three blots identical to the one shown in B were overlaid with native F-actin (C), HMM (D), and tropomyosin (E) and subsequently probed with anti-actin JLA20 mAb (C), anti-myosin MF20 mAb (D), and anti-tropomyosin CH1 mAb (E). In a control experiment, a similar blot was overlaid with the Z-disk protein α-actinin and subsequently probed with an anti-α-actinin mAb. In the last lane of the same blot, 10 ng of purified protein was loaded and served as positive control to the immunodetection system used. An ~100-kDa band was detected that corresponds to α-actinin.

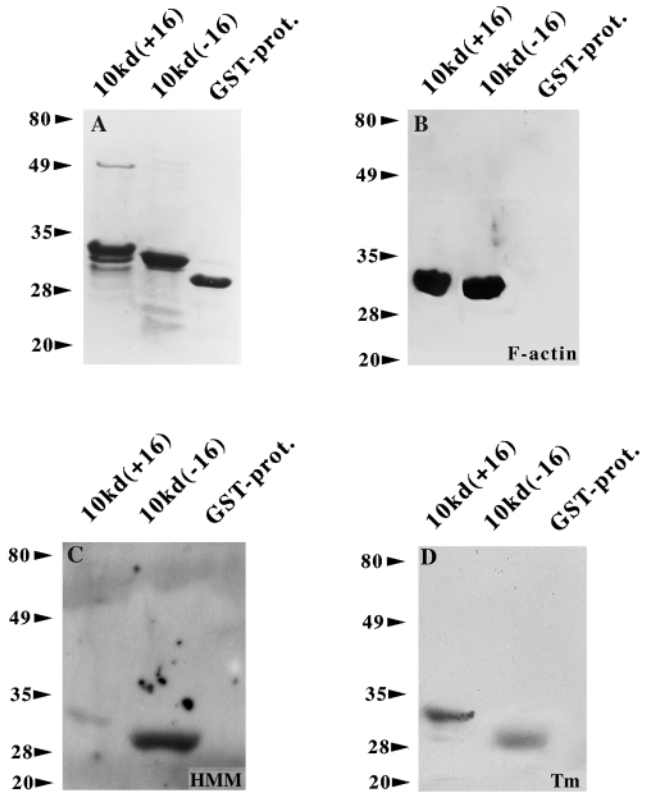

Figure 9. Blot overlay analysis of the interaction between 4.1R-10 kDa domain and F-actin, HMM, and tropomyosin. (A) Similar amounts of GST-10 kDa fusion proteins that either carry or omit exon 16 as well as GST protein alone were subjected to SDS-PAGE and subsequently stained with Coomassie blue. Blots identical to the one shown in A were incubated with purified skeletal muscle F-actin (B), HMM (C), and tropomyosin (D) followed by Western blotting with anti-actin JLA20 mAb (B), anti-myosin MF20 mAb (C), and anti-tropomyosin CH1 mAb (D).

cle homogenates were overlaid with GST-4.1R-10 kDa plus or minus exon 16 as well as control GST protein. The ability of the GST polypeptides to interact with skeletal muscle proteins was subsequently evaluated by Western blotting with the use of anti-GST specific antibodies. A highly specific and strong association between myosin heavy chain, α-actin, and skeletal tropomyosin with either GST-4.1R-10 kDa polypeptide was detected, whereas no interaction was observed in the case of control GST protein (Figure 10A). Notably, GST-4.1R-10 kDa that was missing exon 16 exhibited a higher binding affinity to myosin than GST-4.1R-10 kDa that carried this nucleotide cassette. This finding confirmed our earlier observation (Figures 8D and 9C). No substantial difference was detected in the interaction of α-actin with either of the GST-4.1R-10 kDa polypeptides, as shown in Figures 8C and 9B. Finally, both GST-4.1R-10 kDa variants were able to specifically and efficiently associate in vitro with α- and β-tropomyosin isoforms. It is interesting that the in vivo coIp assays (Figure 6, D, H, and I) indicated that protein 4.1R interacts predominantly with α-tropomyosin, whereas limited amounts of the β isoform were detected in the immunoprecipitate fraction. The ability of 4.1R-10 kDa to associate with either α or β polypeptides in vitro suggests that the 4.1R-binding site on tropomyosin isoforms should share considerable similarity. The identity of the observed immunoreactive bands as myosin, actin, and tropomyosin was confirmed by probing three replica immunoblots with the appropriate antibodies (Figure 10B). Finally, it should be mentioned that a still unidentified skeletal muscle protein of ~110 kDa appeared to strongly and specifically interact with both GST-4.1R-10 kDa polypeptides but not with control GST protein (Figure 10A; the unknown protein is designated X-protein).

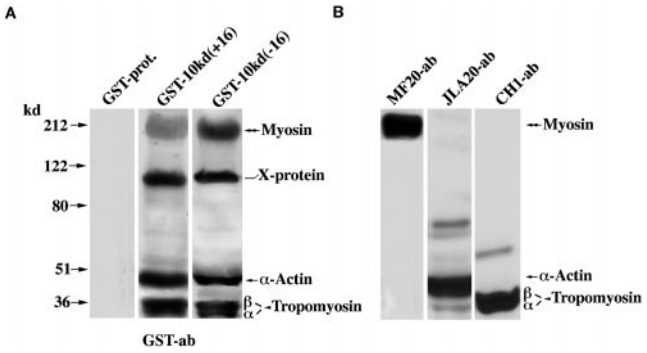

Figure 10. Overlay assays of adult skeletal muscle extracts with GST-4.1R-10 kDa variants. (A) Skeletal muscle homogenates were overlaid with GST-4.1R-10 kDa polypeptides that included or skipped exon 16 and control GST protein, as indicated above the lanes, followed by immunoblotting with anti-GST antibodies. A highly specific and strong association between 4.1R-10 kDa polypeptides and myosin heavy chain, α-actin, and skeletal tropomyosin was detected (immunoreactive proteins are denoted by double arrows, single arrows, and arrowheads, respectively). An additional band of ~110 kDa was also observed that remains unidentified (X-protein). (B) Western blot analysis verified the identity of the immunoreactive bands after probing three replica blots with anti-myosin (MF20-ab), anti-actin (JLA20-ab), and anti-tropomyosin (CH1-ab) antibodies.

DISCUSSION

The prototypical 4.1R is an ~80-kDa phosphoprotein that was identified as a major component of the erythrocyte cytoskeleton, where it contributes to the mechanical integrity and deformability of the cell membrane (Benz, 1993). The presence of 4.1R is not restricted to red blood cells, because variable isoforms have been identified in mammalian and avian nucleated cells (Granger and Lazarides, 1985; Anderson *et al.*, 1988). Nonerythroid 4.1R proteins appear to localize in diverse subcellular compartments, presumably displaying distinct functional activities. In this report, we investigated the number and primary structure of 4.1R isoforms present in adult skeletal muscle and characterized the intracellular distribution of the respective message and protein. Moreover, we demonstrated that 4.1R occurs in vivo in a supramolecular complex with major sarcomeric proteins, including myosin, α-actin, and tropomyosin, and identified the 4.1R domain responsible for each individual interaction.

The extensive and highly complex alternative splicing that the 4.1R gene undergoes, along with the presence of at least two distinct translation initiation codons, prompted us to study the number and primary structure of 4.1R transcripts present in adult skeletal muscle. Exclusive use of the upstream translation initiation codon (AUG-1), residing in exon 2′, was revealed, whereas AUG-2, present in exon 4, does not seem to be used readily in skeletal myofibers. This finding is in agreement with the absence of any prevalent immunoreactive band of ~80 kDa in skeletal muscle homogenates (Huang *et al.*, 1992). The predominant 4.1R isoform constitutes 57% of the total cDNA population and consists of all of the previously identified constitutive exons (Baklouti *et al.*, 1997) plus the alternatively spliced cassettes encoded by exons 2′, 4, 5, 8, 16, 17a, 18, 19, and 20. Four additional 4.1R variants constituted 34% of the total cDNA molecules. In all five 4.1R isoforms, the sequences encoded by exons 2′, 4, 5, 8, 18, 19, and 20 are constitutively included. Thus, the inclusion and/or skipping of exons 16 and 17a appear to be the primary splicing events that take place in the 4.1R messages within skeletal myofibers. The prevalent inclusion of exon 16 (84%) is consistent with a previous observation from our laboratory indicating that among several nonerythroid tissues, skeletal muscle includes this 63-nucleotide motif in the largest amounts (Baklouti *et al.*, 1992, 1997; Discher *et al.*, 1993). On the other hand, exon 17a is a recently identified exon that is highly expressed in striated muscle and liver (Baklouti *et al.*, 1997; Schischmanoff *et al.*, 1997; Kontrogianni-Konstantopoulos *et al.*, 1998). Moreover, it is interesting to note the inclusion of exon 17b in 9% of the total 4.1R cDNAs analyzed. This exon is 450 nucleotides long and is expressed predominantly in epithelial origin tissues and organs, including kidney, intestine, and lung (Schischmanoff *et al.*, 1997).

Two major 4.1R proteins were identified in skeletal muscle homogenates with molecular masses of ~105/110 and ~135 kDa. The identification of these two immunoreactive bands as 4.1R polypeptides is strongly suggested by the use of at least two 4.1R-specific antibodies. Anti-Hp was generated against the unique 4.1R NH_2-terminal Hp extension, which is not shared by any of the recently identified 4.1-like counterparts (Parra *et al.*, 1998). Additionally, anti-E17a was produced against exon 17a, which appears to be present exclusively in the 4.1R gene (Parra *et al.*, 1998). Thus, it is reasonable to assume that both the ~105/110- and ~135-kDa isoforms belong to the family of 4.1R gene products, as opposed to 4.1G, 4.1N, and 4.1B. The identity of the ~105/110- and ~135-kDa isoforms was further verified with the use of another COOH-terminal 4.1R antibody, anti-22/24 kDa, which recognizes epitopes present in exon 19. The different electrophoretic mobilities of the two major 4.1R polypeptides present in skeletal muscle should result from complex alternative splicing events, leading to inclusion and/or skipping of distinct exon combinations as well as extensive posttranslational modifications (i.e., phosphorylation and/or glycosylation) that may take place.

Analysis of the intracellular distribution of 4.1R messages in adult skeletal myofibers revealed the presence of longitudinal signal strands, whereas an intense punctate staining within the myoplasm was observed in cross-sections. This localization pattern may imply a cytoskeletal association of 4.1R transcripts (Russell and Dix, 1992). Notably, skeletal myosin, desmin, and vimentin messages display similar distribution patterns and have been found to associate with the intermyofibrillar cytoskeleton (Issacs and Fulton, 1987; Russell and Dix, 1992).

Protein 4.1R decorates the sarcoplasm in highly periodic transverse striations that are in register with A-bands. Thus, the nonerythroid 4.1R does not reside at the periphery of skeletal myofibers, as its erythroid counterpart does along the erythrocyte membrane, but is positioned within the myoplasm. Recently, novel members of the cytoskeletal $ankyrin_R$ and $ankyrin_G$ families were also shown to display a cytoplasmic reticular distribution that coincided with the sarcoplasmic reticulum of skeletal muscle fibers (Zhou *et al.*, 1997; Kordeli *et al.*, 1998). Additionally, the muscle-specific isoform of β-spectrin was detected within the sarcoplasm of a subpopulation of skeletal myofibers (Porter *et al.*, 1997; Zhou *et al.*, 1998).

The periodic localization pattern of 4.1R message and protein throughout the sarcoplasm may imply a cotranslational assembly process (Fulton *et al.*, 1980; Russell and Dix, 1992). This has been reported for the sarcomeric proteins myosin, vimentin, titin, and tropomyosin that assemble into their cytoskeletal structures during translation (Isaacs and Fulton, 1987; Isaacs *et al.*, 1989a,b; L'Ecuyer *et al.*, 1998). Whether the cotranslational assembly theory holds true for the 4.1R mRNA and protein is unknown. However, it is tempting to presume that the apparent intracellular proximity of the respective message and protein may be indicative of 4.1R protein assembly while it is still being translated.

4.1R and most likely an ~105/110-kDa isoform appear to interact in vivo with the actomyosin sarcomeric cytoskeleton as well as the actin-associated protein tropomyosin. Whether 4.1R associates with the aforementioned contractile proteins simultaneously or in a dynamic manner is a matter of speculation. Nevertheless, the 4.1R-10 kDa domain was shown to be responsible for all of these interactions. This domain consists of the alternatively spliced exon 16 and the majority of the constitutive exon 17. When the binding affinity of native myosin, α-actin, and tropomyosin for 10 kDa was examined in the presence or absence of exon 16, distinct results were obtained for each interaction. 4.1R/α-actin association clearly does not depend on amino acid sequences carried by exon 16. This finding is compatible with observations made by Schischmanoff *et al.* (1995) and Gimm and Mohandas (1999), who reported that residues within exon 17 are the putative binding site of β-actin in the erythrocyte system. On the other hand, the ability of tropomyosin to associate with the 10-kDa domain was diminished (~15%) in the absence of exon 16, suggesting that amino acid sequences within this exon are involved in the 4.1R/tropomyosin interaction along with residues present in the constitutive exon 17. Interestingly, the absence of exon 16 significantly augmented the binding capacity of myosin to the 10-kDa domain. This finding may indicate an inhibitory role for this 21-amino acid cassette in the 4.1R/myosin interaction, probably via repulsive electrostatic interactions or unfavorable conformation of 4.1R protein. Notably, 16% of the total population of skeletal muscle 4.1R messages excluded exon 16 solely or in combination with exon 17a. Whether the 4.1R protein molecules that skip exon 16 preferentially associate in vivo with myosin heavy chain is not known. Nevertheless, it appears that this association is highly dynamic. It presumably depends on the contraction/relaxation state of individual myofibers, and amino acid residues within exon 16 may be key regulators of this process.

The biological significance of protein 4.1R within adult skeletal muscle is uncertain at this time. However, the presence of 4.1R along the A-bands as well as its ability to interact directly with the actomyosin filaments suggest that it may have a structural and/or regulatory role within skeletal myofibers. Some evidence consistent with this notion also comes from earlier in vitro binding studies (Pasternack and Racusen, 1989) indicating that erythroid protein 4.1R partially inhibits the actin-activated Mg^{2+}-ATPase activity of skeletal muscle myosin in a dose-dependent manner. Conceivably, 4.1R may play a pivotal role in the structural organization and maintenance of the contractile apparatus by anchoring the thin and thick myofilaments and modulating their displacements during successive cycles of muscular contraction. Additional work will establish the precise role of the cytoskeletal protein 4.1R in the mechanochemistry of adult skeletal muscle. Our findings clearly implicate 4.1R as an essential component of these processes.

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