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The Novel Skeletal Muscle Sarcoplasmic Reticulum JP-45 Protein

MOLECULAR CLONING, TISSUE DISTRIBUTION, DEVELOPMENTAL EXPRESSION, AND INTERACTION WITH α1.1 SUBUNIT OF THE VOLTAGE-GATED CALCIUM CHANNEL*

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JP-45 is a novel integral protein constituent of the skeletal muscle sarcoplasmic reticulum junctional face membrane. We identified its primary structure from a cDNA clone isolated from a mouse skeletal muscle cDNA library. Mouse skeletal muscle JP-45 displays over 86 and 50% identity with two hypothetical NCBI data base protein sequences from mouse tongue and human muscle, respectively. JP-45 is predicted to have a cytoplasmic domain, a single transmembrane segment followed by an intralumenal domain enriched in positively charged amino acids. Northern and Western blot analyses reveal that the protein is mainly expressed in skeletal muscle. The mRNA encoding JP-45 appears in 17-day-old mouse embryos; expression of the protein peaks during the second month of postnatal development and then decreases ~3-fold during aging. Double immunofluorescence of adult skeletal muscle fibers demonstrates that JP-45 co-localizes with the sarcoplasmic reticulum calcium release channel. Co-immunoprecipitation experiments with a monoclonal antibody against JP-45 show that JP-45 interacts with the α1.1 subunit voltage-gated calcium channel and calsequestrin. These results are consistent with the localization of JP-45 in the junctional sarcoplasmic reticulum and with its involvement in the molecular mechanism underlying skeletal muscle excitation-contraction coupling.

The sarcoplasmic reticulum is an intracellular membrane compartment that controls the intracellular calcium concentration and, therefore, plays an important role in excitation-contraction (E-C)[1] coupling (1–3). The anatomical site of E-C coupling is the triad, an intracellular synapse formed by the association of two membrane compartments: the transverse tubules, which are an invagination of the plasmalemma, and the terminal cisternae (2–4). The portion of terminal cisternae facing the transverse tubules is called the junctional face membrane. Two major protein constituents of the triad membranes have been identified and shown to play a crucial role in E-C coupling: the skeletal muscle voltage-gated calcium channel dihydropyridine receptors (DHPRs) localized in T-tubule membrane and the ryanodine receptors (RyRs) localized in the sarcoplasmic reticulum junctional face membrane (5–9). In skeletal muscle the DHPRs respond to transverse tubule depolarization by sensing the voltage variation and induce calcium release from the skeletal muscle sarcoplasmic reticulum (SR) via direct activation of the RyR (8, 9).

Besides RyRs, the sarcoplasmic reticulum junctional face membrane contains a number of proteins, including triadin, calsequestrin, and junctin (10) as well as other known and unknown proteins (11–16). Because of their localization, proteins present within this membrane are likely to be involved in E-C coupling, and a defect in their function could potentially lead to alterations of the calcium release process and/or intracellular homeostasis leading to neuromuscular diseases (16–21).

We recently identified JP-45, an integral membrane polypeptide of 45 kDa of skeletal muscle junctional face membrane (22). In the present investigation, we extended our studies and carried out the molecular characterization of JP-45. Our results show that this polypeptide is mainly expressed in skeletal muscle and that its level of expression is down-regulated during aging. In addition, we show that it interacts with two key proteins involved in skeletal muscle E-C coupling, namely the α1.1 subunit of the DHPR and calsequestrin.

EXPERIMENTAL PROCEDURES

Materials

Keyhole-limpet hemocyanin (KLH) conjugation kit, MPL+TDM adjuvant, CHAPS, and protein G-Sepharose were from Sigma; glutathione-Sepharose 4B, pGex plasmid, nitrocellulose membranes, and [^{32}P]dCTP were from Amersham Biosciences. Goat anti-α 1.1 subunit of the skeletal muscle DHPR polyclonal antibodies were from Santa Cruz Biotechnology Inc. RNA isolation kit was from Biotecx Laboratories (Houston, TX). Anti-calsequestrin antibodies were raised in guinea pigs and previously characterized (23). Fluorescein isothiocyanate- and TRITC-conjugated secondary antibodies were from DAKO. Sheep anti-RyR antibodies were from Upstate Biotechnology (Lake Placid, NY). Peroxidase-conjugated protein G and protein A were from Bio-Rad. Peroxidase-conjugated secondary antibodies, antiprotease mixture, the chemiluminescence kit, restriction enzymes, random primed DNA labeling kit, DNA-digoxigenin labeling kit, and anti-digoxigenin peroxi-

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[1] The abbreviations used are: E-C, excitation-contraction; DHPR, dihydropyridine receptor; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; TRITC, tetramethylrhodamine isothiocyanate; mAb, monoclonal antibody; GFP, green fluorescence protein.

dase-conjugated antibodies were from Roche Applied Science and New England Biolabs. Mouse skeletal muscle UNI-ZAP XR cDNA library and the cDNA synthesis kit were from Stratagene. Mouse multiple-tissue Northern blot, RNA master blot, and the pEGFP plasmids were from Clontech. All other chemicals were of reagent grade.

Methods

cDNA Cloning—We isolated and purified total RNA from Balb/c mice skeletal muscles using an RNA isolation kit from Biotecx Laboratories following the instructions provided by the manufacturer. Total RNA was converted into cDNA using a cDNA synthesis kit from Roche Applied Science using oligo$(dT)_{15}$ primers, following the instructions provided by the manufacturer. To amplify the cDNA specific for JP-45, we first searched the NCBI data base for sequences matching the polypeptide sequences we obtained from rabbit skeletal muscle JP-45 (22). The available sequences matched a hypothetical mouse tongue protein (*Mus musculus*; NCBI accession number AK009578). Based on the primary sequence of this hypothetical protein, we designed a set of primers to amplify the portion of JP-45 encompassing almost all of the peptide sequences we had obtained by tryptic and CNBr digestion of the native protein. The primers used to amplify the cDNA corresponding to JP-45 cDNA were 5′-AGAATTCCCGCCCCCAAGACGCCAAGC-3′ and 5′-AGAATTCACGGCCTTTATTTGCTGGTGC-3′ for forward and reverse reactions, respectively. The *Eco*RI restriction enzyme sequence was added to facilitate subsequent subcloning. 100 ng of mouse skeletal cDNA were amplified in a PerkinElmer Life Sciences GeneAmp 2400 PCR system under the following conditions: 5 min at 95 °C, followed by 35 cycles of 40 s annealing at 63 °C, 45 s extension at 72 °C and 30 s denaturation at 92 °C, and a final elongation step of 4 min at 72 °C. The nature and identity of the amplified cDNA was verified by direct sequencing (Microsynth AG, Balgach, Switzerland). The PCR-amplified cDNA fragment was labeled and used to screen a mouse skeletal muscle UNI-ZAP XR cDNA library as previously described (24).

Northern Blot Analysis—Northern blot filters containing 2 μg of poly$(A)^+$ RNA from eight different mouse tissues and a dot blot containing poly$(A)^+$ RNA from mouse embryos were hybridized with a ^{32}P-labeled cDNA probe obtained from the full-length clone, 193C-1, as previously described (24).

Monoclonal Antibody Production—Mouse monoclonal antibodies were generated against a synthetic peptide designed on a sequence derived from a sequence of rabbit skeletal muscle JP-45. The peptide was conjugated to the keyhole-limpet hemocyanin carrier and used to immunize Balb/c mice. Splenocytes from the immunized mice were fused with tissue culture-grown myeloma cells (AGX8) and hybridomas selected in hypoxanthine/aminopterin/thymidine (HAT) medium. Hybridoma cells were identified and tested for the presence of anti-JP-45 antibodies by Western blot analysis on rabbit terminal cisternae (22, 25).

Expression of JP-45 in Mammalian Cells and Tissues—The entire coding sequence of JP-45 was cloned in-frame into the pEGFP-C1 vector by amplifying the cDNA by PCR using the following forward and reverse primers, 5′-AGAATTCTATGACTACCAGAGGCCTGG-3′ and 5′-AGGTACCACACAGGATCAGTC-3′, respectively, under the same conditions as described above. Expression of the recombinant protein was monitored by fluorescence microscopy on COS-7 cells (Lipofectin) transfected either with pEGFP alone or pEGFP-JP-45, using an Axiophot Zeiss fluorescent microscope equipped with NeoFLUAR objective (×63 oil, N.A. = 1.40) as previously described (24).

Chemical skinning of soleus rat muscles was performed as previously described (26). Single fibers were then isolated under a dissecting microscope, transferred into a Petri dish chamber containing 0.5 ml of a relaxing solution (170 mM K-propionate, 2.5 mM Mg-propionate, 5 mM K_2EGTA, 10 mM imidazole, pH 7.0, 5 mM K_2Na_2ATP) and inserted, in a slightly stretched position, between two clamps. The procedure for immunofluorescence staining was initiated by incubating fibers for 1 h in the relaxing solution containing 1% Triton X-100 (RS-T) and 10% goat non-immune serum. All following steps, both washings and incubation with primary or secondary antibodies, were carried out by using the RS-T. Fibers were first incubated for 90 min with the anti-JP 45-kDa antibody (2 μg/ml), washed three times, then incubated for 40 min with the goat anti-mouse IgG TRITC-conjugated secondary antibody (1:100) in the presence of 1% rat non-immune serum. After washing, fibers were then incubated with a sheep polyclonal antibody specific for the rabbit skeletal muscle ryanodine receptor (diluted 1:200). After 90 min, fibers were washed three times and incubated 40 min with goat anti-sheep IgG fluorescein isothiocyanate-conjugated secondary antibody (1:100). After the final wash, fibers were removed from clamps, mounted in an Elvanol solution on glass slides, and observed with a Leica DMRB fluorescent microscope equipped with a CCD camera. Captured images were analyzed with image processing software (Casti Imaging, Padova, Italy).

TSA201 cells with the pEGFP and pEGFP-JP-45 constructs were transfected; 48 h post-transfection, cells were harvested and crude microsomal membranes prepared by differential centrifugation steps ($3{,}000 \times g_{max}$, $15{,}000 \times g_{max}$, $150{,}000 \times g_{max}$). Integral microsomal membrane components were separated from their soluble counterpart by high salt wash (0.6 M KCl) and extraction with 100 Na_2CO_3 at alkali pH as previously described (11). Microsomes were also prepared from the brain, tongue, heart, liver, spleen, kidney, and skeletal muscles of 2-month-old Balb/c mice as well as from the skeletal muscles of 4-day-, 2-month-, 10-month-, and 14.5-month-old Balb/c mice, as described previously (22, 24). For the latter preparations, which were used to examine the changes that occur in JP-45 during development, four different preparations from at least four different mice were used.

Electrophoresis and Immunological Staining—SDS-PAGE and indirect immunostaining were carried out as previously described. Protein concentration was determined as described by Bradford (31), using bovine serum albumin as standard (22, 24).

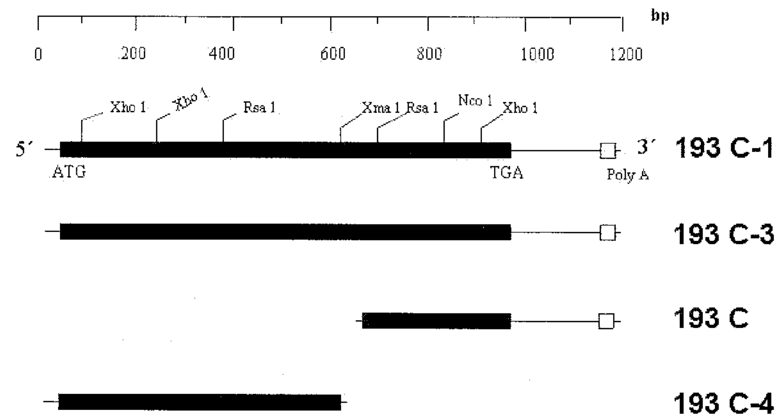
Co-immunoprecipitation Assay—Vesicles derived from the light (R1) and heavy (R4) skeletal muscle membrane fractions and from triads were prepared as previously described (25). Proteins were solubilized (at a final concentration of 1 mg/ml) in a buffer composed of 200 mM NaCl, 50 mM Tris-HCl, pH 8.5, 1 mM dithiothreitol, 1% CHAPS, and anti-proteases for 30 min at room temperature. Soluble proteins were obtained by centrifugation at $150{,}000 \times g$ for 30 min at 4 °C and then were diluted 10 times with a buffer containing anti-proteases mixture, 200 mM NaCl, 50 mM Tris-HCl, pH 8.5, and finally incubated with 1 μg of anti-JP-45 monoclonal antibodies for 1 h. Protein G-Sepharose previously equilibrated with 10 mM Tris-HCl, 150 mM NaCl (pH 8.0), 0.001% Tween (TBST) was added to precipitate the immunocomplex. The immunoprecipitated proteins were separated by electrophoresis in a 10% polyacrylamide gel and then transferred onto nitrocellulose membranes. The proteins pulled down with JP-45 were detected by immunoblot analysis using a panel of antibodies specific for proteins found in triad membranes.

Data Analysis—For group comparison the one-way analysis of variance test was used.

RESULTS AND DISCUSSION

cDNA Cloning, Sequence Analysis, and Expression of Recombinant JP-45 Protein—To obtain the cDNA encoding JP-45, we matched the amino acid sequence information obtained from the peptides generated by proteolytic treatment of rabbit skeletal muscle JP-45 (22) with sequence information available from data bases. Our data matched a hypothetical sequence from mouse (*Mus musculus*; NCBI accession number AK009578) tongue. Based on the latter nucleotide sequence, we designed a set of primers and amplified a 710-bp cDNA fragment (corresponding to nucleotides 590–1319 of the full-length cDNA encoding JP-45). The nucleotide sequence of the PCR fragment matched that of sequence AK009578. The PCR fragment was then ^{32}P-labeled and used to screen under high stringency conditions (three washes at 65 °C, 0.1% SDS, 0.2 × SSCP) a commercially available mouse skeletal muscle cDNA library. Of $\sim 1 \times 10^6$ plaques that were screened, a total of four positive clones were pulled out, plaque-purified, and sequenced (Fig. 1, *upper panel*, *193C*, *193C-1*, *193C-3*, and 193C-4). Two overlapping clones (193C-1, of 1160 bp, and 193C-3, of 1158 bp) contain an identical open reading frame of 996 bp corresponding to a 332-amino acid-long polypeptide. Both clones 193C-1 and 193C-3 possess 66 nucleotides in the 5′-untranslated region upstream from the first initiation codon. A single polyadenylation signal, AAATAA, is observed 18 nucleotides upstream from the poly$(A)^+$ tail (not shown).

Northern blot analysis of tissues from eight different adult mice revealed a strong hybridizing transcript of ~1.16 kb only in skeletal muscle; the size of the transcript matches the size of clone 193C-1 (Fig. 2, *upper panel*). In addition, the transcript encoding JP-45 first appears in 17-day-old embryo mice. Multiple sequence alignment shows that the sequence of the mouse

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MouseSkMuscJP-45          1 --MTTRGLEDLDGGLGSCLPSDDLPFLEEPASGRRPESKARGTSRRADSSDWTHVLQDPV
MouseTongue               1 ---------------------------------------MLGTESWLP---ACWVLQDPV
HumanMusc                 1 MSMTTRGWEDLDGGLGSCQALDDHSALAET---Q--EDKASGTPRLADS--ATVPHDSQV
RabbitSkMuscpepJP-45      1 ------------------------------------------------------------
consensus                 1   mttrg edldgglgsc   dd   l e        e ka gt r ads  at vlqdpv

MouseSkMuscJP-45         59 AAG-AGDAGLKKMEKELAGKESTAG-----KAGTSPRIVPARRKPQAPPPLQPPPPPLQP
MouseTongue              19 AAG-AGDAGLKKMEKELAGKESTAG-----KAGTSPRIVPARRKPQAPPPLQPPPPPLQP
HumanMusc                54 AEGPSVDTRPKKMEKEPAAKGTPSTGKERLKAGASPRSVPARKKAQTAPPLQPPPP----
RabbitSkMuscpepJP-45      1 ------------------------------------------------------------
consensus                61 aag agdaglkkmekelagkestag     kagtsprivparrkpqappplqpppppLqp

MouseSkMuscJP-45        113 PPRTPSDDLPWGDLTLNKCLVLASLVALLGSALQLCRDAVAGEVVA---APHPWVPPSSP
MouseTongue              73 PPRTPSDDLPWGDLTLNKCLVLASLVALLGSALQLCRDAVAGEVVA---APHPWVPPSSP
HumanMusc               110 -PPALSEELPWGDLSLNKCLVLASLVALLGSAFQLCRDAVPGEAALQARVPEPWVPPSSA
RabbitSkMuscpepJP-45      1 ------------------------------------------------------------
consensus               121 pprtpsddlpwgdltlnkclvlaslvallgsalqlcrdavagevva   aphpwvppssp

MouseSkMuscJP-45        170 PKKEASPAPKPPVLVSPSGSPQPKPGPPQARMQDEPELPGSPEATETRVERGGSIS-EAS
MouseTongue             130 PKKEASPAPKPPVLVSPSGSPQPKPGPPQARMQDEPELPGSPEATETRVERGGSIS-EAS
HumanMusc               169 PKEPSSPLPKFEAQAPPSSPPAPK-----AEASVRPKSPGSREAAENDEEEPGEASGEAV
RabbitSkMuscpepJP-45      1 ------------------------------------------------------------
consensus               181 pkkeaspapkppvlvspsgspqpkpgppqarmqdepelpgspeatetrverggsis eas

MouseSkMuscJP-45        229 GEESVPLGDRGSQEKPRKE-KPSKGEKLKKEKPRREKPRREDKSQVTGEPRQSLPRRWEA
MouseTongue             189 GEESVPLGDRGSQEKPRKE-KPSKGEKLKKEKPRREKPRREDKSQVTGEPRQSLPRRWEA
HumanMusc               224 REDRVTLGDRGPKEKPRKEGKPRKEKPRKEERPKKEKPRKEKKPRAAREPRKALPQRWES
RabbitSkMuscpepJP-45      1 ------------------------------------------------------------
consensus               241 geesvplgdrgsqekprke kpskgeklkkekprrekprredksqvtgeprqslprrwea

MouseSkMuscJP-45        288 REGGRRPWGRDSRDLLEHGKLQAWAPRRRHDRDDRPQKR----GKGRD
MouseTongue             248 REGGRRPWGRDSRDLLEHGKLQAWAPRRRHDRDDRPQKR----GKGRD
HumanMusc               284 REGGKRPWARDSRDAEPK-KKQAWVSPRRPDEDQRPGSKQKLRAGKGRD
RabbitSkMuscpepJP-45      1 REGGRGPWARDSRDPGRS-KRQARAPRR-HDRDD---------------
consensus               301 REGGrrPWgRDSRDllehgKlQAwaprRrhDrddrprqkr    gkgrd
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FIG. 1. **Predicted primary sequence of mouse skeletal muscle JP-45 and comparison of its amino acid sequence to that of mouse tongue, human muscle, and rabbit skeletal muscle.** *Top*, restriction map of the full-length cDNA clone (*193C-1*) encoding JP-45 and of three other partial clones (*193C-3*, *193C*, and *193C-4*) also isolated from the mouse skeletal muscle library. The *dark shaded rectangles* represent the open reading frame; the *small white box* represents the poly(A+) tail. *Bottom*, multiple sequence alignment was carried out using the ClustalW computer program available from the Swiss node of the European Molecular Biology network. *Black boxes* indicate identical residues; *gray boxes* indicate conserved residues. *Underlined sequences* match the peptide sequences obtained from proteolytic treatment of JP-45.

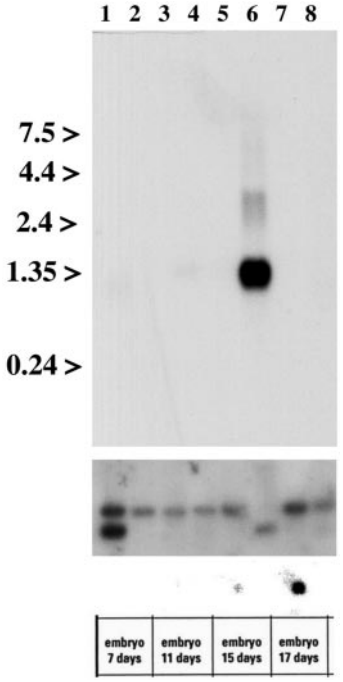

FIG. 2. **Tissue and embryological expression of JP-45.** *Top*, 2 μg of poly(A)$^+$ RNA per lane from eight different mouse tissues were hybridized to a ^{32}P-labeled cDNA probe encompassing nucleotides 19–1047 of mouse skeletal muscle JP-45. *Lane 1*, heart; *lane 2*, brain; *lane 3*, spleen; *lane 4*, lung; *lane 5*, liver; *lane 6*, skeletal muscle; *lane 7*, kidney; and *lane 8*, testes. *Center*, the blot was hybridized with β-actin cDNA probe as control. *Bottom*, 1 μg of poly(A)$^+$ RNA from 7-, 11-, 15-, and 17-day-old mouse embryos was hybridized to a ^{32}P-labeled cDNA probe encompassing nucleotides 19–1047 of mouse skeletal muscle JP-45.

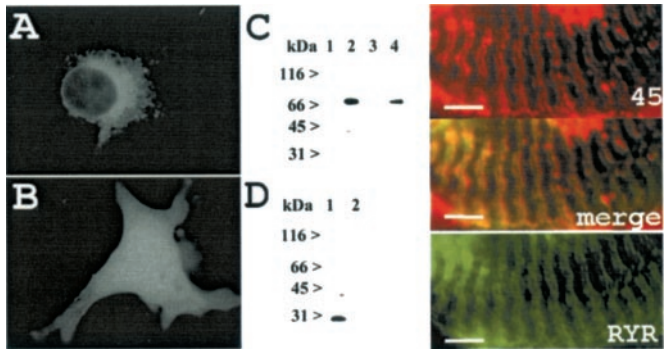

FIG. 3. **Cellular distribution of JP-45.** *A* and *B*, cells were examined for GFP fluorescence with a Zeiss Axiovert microscope using a ×63 NeoFLUOR objective (1.4 N.A., excitation 480 nm; magnification ×1000). *C* and *D*, microsomes from TSA201 cells transfected with the pEGFP-JP-45 and pEGFP plasmids were washed with high salt and 100 mM Na_2CO_3. Soluble proteins and proteins loosely associated with the membranes (*lanes 1* and *3*) were separated from integral membrane proteins (*lanes 2* and *4*) by differential centrifugation. 20 μg of protein were loaded per lane, separated on a 10% SDS-PAGE, blotted onto nitrocellulose, and probed with anti-GFP antibodies (*panels C* and *D*, *lanes 1* and *2*) or anti-JP-45 antibodies (*panel C*, *lanes 3* and *4*). *Right panels*, double immunofluorescence staining of chemically skinned single fibers from rat soleus muscle with the monoclonal anti-JP-45 antibody (*JP45*), a polyclonal anti-RyR antibody (*RYR*). The merged image (*merge*) shows the overlap of the staining of the two antibodies. *Scale bar* = 10 μm.

skeletal muscle cDNA clone encodes a protein that displays 86 and 50% identity with hypothetical proteins from mouse tongue and human muscle (Fig. 1, *lower panel*). The NH_2-terminal sequence of mouse skeletal muscle JP-45 is similar to its human counterpart but diverges from that of mouse tongue. The mouse skeletal muscle JP-45 and the hypothetical tongue protein are identical from Val at position 54 until the end of the protein. The peptide sequence obtained from proteolytic treatment of rabbit skeletal muscle JP-45 that was used to generate the anti-JP-45 mAb is localized at the COOH-terminal part of the proteins and displays 71 and 50% identity with the corresponding mouse and human sequences, respectively.

The deduced primary sequence of mouse skeletal muscle JP-45 predicts a protein of 332 amino acids with a molecular mass of 36 kDa. Transmembrane prediction analysis (TMpred, www.ch.EMBnet.org) suggests the possibility of one transmembrane domain, between residues 126–148, which is 100 and 91% identical to the hypothetical mouse tongue and human muscle sequences, respectively. We confirmed that JP-45 is an integral membrane protein by cellular and biochemical approaches. When COS-7 cells were transfected with the pEGFP-JP-45 construct, the green fluorescence was excluded from the nuclei and showed a pattern of fluorescence compatible with its distribution within intracellular membranes (Fig. 3*A*). On the other hand, when cells were transfected with the empty pEGFP vector, the fluorescence was evenly distributed throughout the cytoplasm and nuclei (Fig. 3*B*). These results are consistent with localization of the recombinant EGFP-JP-45 fusion protein in the sarco(endo)plasmic reticulum membrane. The conclusion is also supported by Western blot analysis of KCl-washed, sodium carbonate-treated membrane fraction isolated by differential centrifugation from TSA201 cells transfected with pEGFP-JP-45. A band having a molecular mass of about 70 kDa (27 kDa corresponding to the mass of GFP, plus 45 kDa corresponding to the apparent mass of JP-45) was immunodecorated with anti-GFP (Fig. 3*C*, *lanes 1* and *2*) and anti-JP-45 antibodies (Fig. 3*C*, *lanes 3* and *4*) and is clearly present in the membranes after Na_2CO_3 extraction (Fig. 3*C*, *lanes 2* and *4*) and absent from the soluble fraction (Fig. 3*C*, *lanes 1* and *3*). This result is consistent with the conclusion that the punctuated fluorescent pattern of living COS-7 is not because of aggregates of denatured recombinant protein within ER membranes. TSA201 cells transfected with the empty pEGFP vector display an immunoreactive band of ~27 kDa only in the soluble fraction (Fig. 3*D*, *lane 1*).

To localize JP-45 in native muscle fibers, we performed double immunofluorescence experiments on adult rat muscle fibers using our anti-JP-45 monoclonal antibodies and commercially available anti-RyR receptor antibodies. Staining with either antibody shows a typical triadic striation pattern (Fig. 3, *right panels*, *JP45* and *RyR*). The merged image supports the view that JP-45 and ryanodine receptor co-localize in triad membranes.

Mouse skeletal muscle JP-45 contains 112 charged amino acid residues of a total of 332; the lumenal domain alone contains 76 (of 112) charged residues, 47 of which are basic and 30 acidic, giving the polypeptide a predicted pI of 9.77. The higher apparent molecular mass of the JP-45 observed in the Laemmli gel system could be because of the basic nature of the protein, as already described for triadin, another basic SR protein. The protein contains eight putative protein kinase As, three protein kinase Cs, and five CaMKII phosphorylation sites.

Tissue Distribution and Developmental Expression of JP-45—To investigate the tissue distribution at the protein level we used the monoclonal antibody raised against a synthetic peptide derived from the rabbit skeletal muscle JP-45 sequence (Fig. 1*B*, *RabbitSk-Muscpep*). It should be pointed out that this mAb immunodecorated a glutathione *S*-transferase fusion protein encompassing the COOH-terminal domain of the mouse skeletal muscle JP-45 (not shown), indicating that its epitope is shared by the rabbit and mouse proteins. The presence of the JP-45 protein product in the microsomal fractions from different mouse tissues was verified by

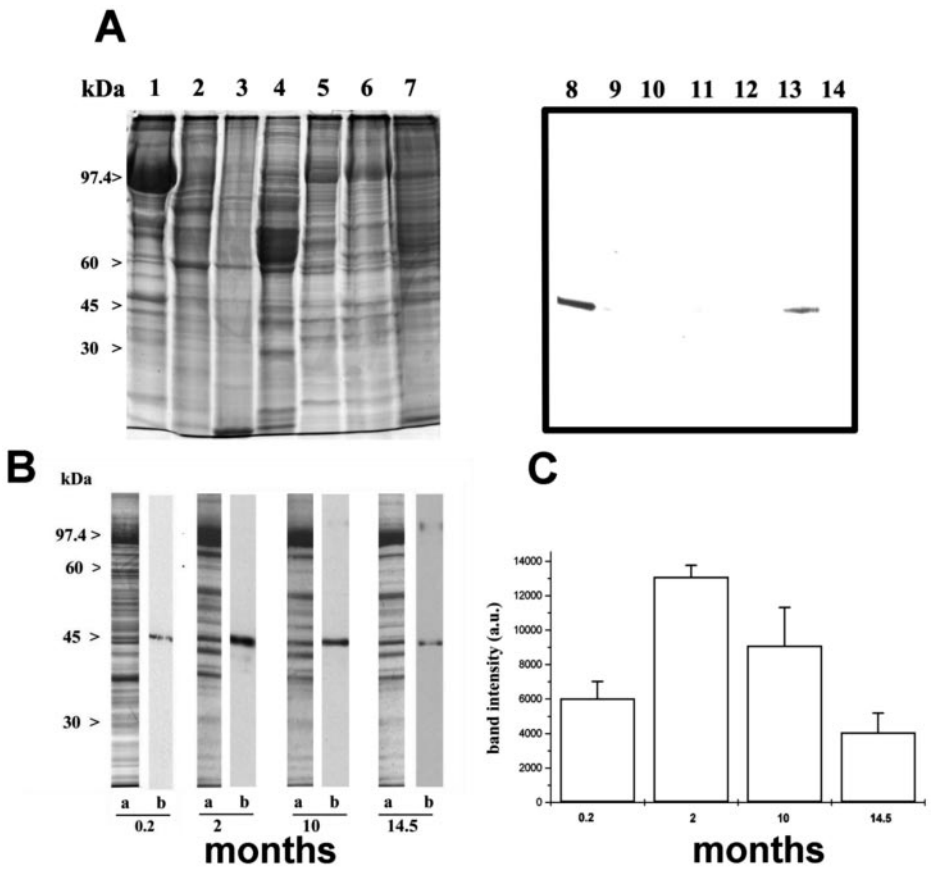

FIG. 4. **Western blot analysis showing tissue distribution and developmental expression of JP-45 protein.** *A*, gel Coomassie Brilliant Blue staining (*lanes 1–7*) or JP-45 immunostaining (*lanes 8–14*) of 10 μg of microsomes from different tissues obtained from Balb/c mice. *Lanes 1* and *8*, skeletal muscle; *lanes 2* and *9*, brain; *lanes 3* and *10*, heart; *lanes 4* and *11*, liver; *lanes 5* and *12*, spleen; *lanes 6* and *13*, kidney; *lanes 7* and *14*, tongue. *B*, total microsomes obtained from Balb/c mouse muscles of the indicated age groups were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (*a*) or with anti-JP-45 mAb (*b*). Ten micrograms of protein were loaded per lane. *C*, densitometric scanning of the intensity of the JP-45 immunopositive band from different muscle preparations. The intensity of the JP-45 immunopositive band was compared for different mice of different age groups using the analysis of variance test ($p < 0.0036$).

Western blot analysis. An immunoreactive band is present in total mouse microsomal membranes isolated from skeletal muscle and tongue (Fig. 4*A*, *lanes 8* and *13*) but absent from microsomal membranes of kidney, spleen, liver, heart, and brain (Fig. 4*A*, *lanes 9–12* and *14*, respectively).

We next examined whether the expression of JP-45 changes during development by comparing its content in microsomal membranes from skeletal muscles obtained from mice of different age groups (0.2, 2, 10, and 14.5 months). Four separate SR preparations from at least four different animals of each age group were made and their content of JP-45 assessed. A clear increase in the total content of JP-45 was shown during the early phase of postnatal development, and the amount of protein peaks at ~2 months (Fig. 4*B*) and thereafter in 14.5-month-old mice decreases 3.2-fold (analysis of variance $p < 0.0036$) (Fig. 4*C*). The average mouse life span is 2 years; thus, it appears that JP-45 is down-regulated during aging of mouse skeletal muscle (27, 28).

Protein-Protein Interactions of JP-45 with Skeletal Muscle Sarcotubular Membrane Proteins—The junctional face membrane of sarcoplasmic reticulum contains proteins that play a crucial role in the mechanism of E-C coupling. The selective localization of JP-45 in the junctional face membrane suggests that this protein may also be involved in the mechanism underlying E-C coupling by interacting with other junctional face membrane protein constituents. To test this hypothesis, we carried out co-immunoprecipitation experiments with the anti-JP-45 mAb. The specificity of the anti-JP-45 mAb was assayed against vesicles from light R1, R2, and heavy R4 muscle membrane fractions (25, 29, 30). A single immunoreactive band migrating as a polypeptide of ~45 kDa is present in vesicles derived from light SR but is clearly enriched in the heavy R4 membrane fraction (Fig. 5*A*). To determine whether JP-45 forms a complex with other proteins present in the sarcoplasmic reticulum, we performed co-immunoprecipitation experiments. Vesicles from light R1 and heavy R4 muscle membrane fractions were solubilized with CHAPS, and the proteins contained in the clarified supernatant were incubated with anti-JP-45 antibodies followed by incubation with protein G-Sepharose beads. The co-immunoprecipitated proteins were analyzed by Western blotting using antibodies directed against protein constituents of the triad membranes. Neither triadin nor the RyR could be co-immunoprecipitated by the JP-45 antibodies (results not shown). In the light R1 sarcoplasmic reticulum fraction, anti-α1.1 subunit of the voltage-gated calcium channel antibodies stained a single polypeptide of ~200 kDa (Fig. 5*B*, *lane 1*). When the proteins present in the co-immunoprecipitated complex were probed with the anti-α1.1 subunit of the voltage-gated calcium channel antibodies, an immunoreactive band with an electrophoretic mobility identical to that present in the light R1 fraction was obtained (Fig. 5*B*, *lane 3*). In control experiments in which the light R1 sarcoplasmic reticulum fraction was incubated with the Sepharose beads in the absence of anti-JP-45 mAb (Fig. 5*B*, *lane 4*) no immunoreactivity was obtained, confirming the specificity of the interaction (Fig. 5*B*, *lane 4*). In another control, we incubated the immunoprecipitating anti-JP-45 antibodies with the protein G-Sepharose beads in the absence of sarcoplasmic reticulum fraction; under these conditions no immunodecorated band was observed (Fig. 5*B*, *lane 2*).

A similar set of experiments was performed to verify whether calsequestrin was part of the immunoprecipitated complex. The specificity of the anti-calsequestrin antibodies is shown in Fig. 5*C*, *lane 1*: a polypeptide of ~60 kDa is recognized by the antibodies in the heavy sarcoplasmic reticulum R4 fraction. When the proteins present in the co-immunoprecipitated complex were probed with the anti-calsequestrin antibodies, an immunoreactive band of ~60 kDa was present (Fig. 5*C*, *lane 3*).

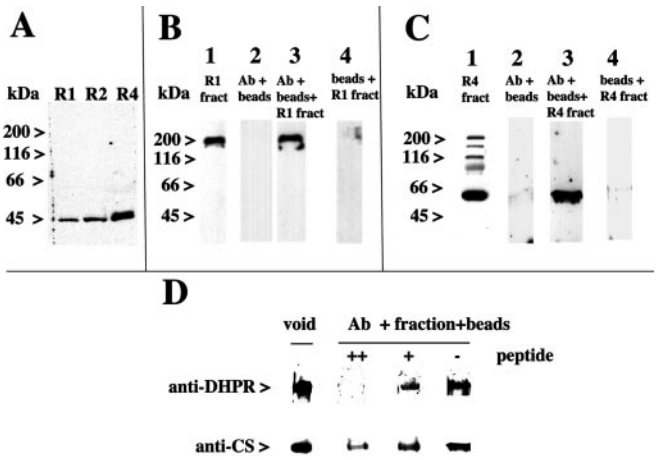

FIG. 5. **Immunoprecipitation of solubilized light and heavy SR fractions.** *A*, immunoreactivity of the anti-JP-45 monoclonal antibodies. Twenty micrograms of rabbit skeletal muscle R1, R2, and R4 sarcoplasmic reticulum-derived vesicles were separated on a 10% SDS PAGE and probed with 0.5 μg/ml anti-JP-45 mAb. The immune reaction was visualized with anti-mouse peroxidase-conjugated antibodies followed by chemiluminescence. *B* and *C*, thirty micrograms of rabbit skeletal muscle R1 or R4 sarcoplasmic reticulum-derived vesicles were separated on a 10% SDS PAGE and probed with 0.5 μg/ml anti-α1.1 subunit of the voltage-gated calcium channel (*panel B*, *lane 1*) and anti-calsequestrin (*panel C*, *lane 1*) antibodies. CHAPS-solubilized vesicles were immunoprecipitated with 1 μg of anti-JP-45 mAb as described under "Experimental Procedures." The pulled-down proteins were separated in a 10% SDS PAGE, blotted onto nitrocellulose, and probed with anti-α1.1 subunit of the voltage-gated calcium channel or anti-calsequestrin antibodies. *Ab+beads*, control; no SR-derived proteins were present. *Ab+beads+fract*, proteins bound to the beads and forming an immune complex. *Beads+fract*, control, no antibody present. The immune reaction was visualized with protein G or protein A peroxidase conjugates, respectively, followed by chemiluminescence. Conditions were as described under "Experimental Procedures." *D*, conditions as described in *panels B* and *C* except that co-immunoprecipitation was carried out in the absence (− peptide) or presence (++, 1.3-mg peptide; +, 0.9-mg peptide) of increasing concentrations of immunogenic peptide (*Ab+beads+fraction* proteins bound and forming an immune complex). *Void*, proteins not bound to the Ab+beads. The *blots* were probed with anti-α1.1. subunit of the voltage-gated calcium channel and anti-calsequestrin antibodies as described for *panels B* and *C*.

Control experiments in which no sarcoplasmic reticulum-derived fraction or no immunoprecipitating anti-JP-45 antibodies were added (Fig. 5*C*, *lanes 2* and *4*, respectively) demonstrate the lack of immunoreactive bands, indicating the specificity of the interaction.

The specificity of the co-immunoprecipitation reaction was verified using a different muscle membrane preparation enriched in triads (7) as well as by preincubating the precipitating anti-JP-45 antibodies with their immunogenic peptide. The anti-JP-45 mAb co-immunoprecipitated the α1.1 subunit of the voltage-gated calcium channel from CHAPS-solubilized triad membranes (Fig. 5*D*) and calsequestrin from R1 CHAPS-solubilized vesicles. At least three concentrations of precipitating anti-JP-45 antibodies were used (0.5, 0.8, and 1 μg protein) and under our experimental conditions 1 μg yielded the best results. To further verify that the immunoprecipitation was not because of nonspecific effects, we carried out the co-immunoprecipitation experiment in the presence of increasing concentrations of the competing soluble immunogenic peptide (++, 1.3-mg peptide; +, 0.9-mg peptide; −, no peptide). Our results show that by blocking the interaction of the antibody with its antigen, we progressively inhibit precipitation of the immunocomplex.

In conclusion, the results reported here provide unequivocal biochemical evidence that the novel JP-45 protein co-localizes with RyR and interacts with the α1.1 subunit of the voltage-gated calcium channel DHPR and the sarcoplasmic reticulum calcium-binding protein calsequestrin, suggesting an important role of this protein in the basic mechanisms underlying E-C coupling in normal and aged skeletal muscle.

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